IMMUNE PATHOLOGY OF A VIRAL INFECTION OF THE FOETAL LAMB

A Thesis submitted for the Degree of

DOCTOR OF PHILOSOPHY

in the

Australian National University

by

SUSAN JANE McCLURE

Department of Immunology,

John Curtin School of Medical Research,

Australian National University,

Canberra, Australia.

January, 1985.

STATEMENT

Dr. Peter McCullagh, Dr. Max Simpson-Morgan and Dr. Wendy Trevella assisted me in performing some of the foetal surgery; Miss Wendy Hughes and Miss Lesley Maxwell assisted me with the histology; with these exceptions, the experiments described in and the writing of this thesis were done entirely by myself.

S. J. McCLURE

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

I am grateful to the Head of the Department of Immunology, of the John Curtin School of Medical Research, Professor Bede Morris, for the opportunity to undertake this study, the Australian National University for the award of a Postgraduate Scholarship, and the A.N.U. and C.S.I.R.O. for collaborative research fund support.

I should like to thank Mrs. Pam Ferris of the Department of Microbiology, and the staff of the Animal Breeding Establishment, the operating theatre and the Histology Unit for their valuable assistance throughout this work.

Finally, I particularly wish to thank my supervisor, Dr. Peter McCullagh, for his encouragement and advice throughout the duration of this work and in the preparation of this thesis.

LIST OF TABLES

Page

Chapter 7

LIST OF FIGURES

LIST OF PLATES

GLOSSARY OF ABBREVIATIONS

PL	:	-propiolactone
BSA	:	bovine serum albumin
BSS	:	balanced salt solution
Con A	:	concanavalin A
Cpm	:	counts per minute
EL	:	efferent lymphocytes
FCS	:	foetal calf serum
Ig	:	immunoglobulin
IgG	:	immunoglobulin G
IgM	:	immunoglobulin M
I/P	:	intraperitoneal
I/V	:	intravenous
LPS	:	lipopolysaccharide
LTA	:	lymphocyte transformation assay
MLC	:	mixed lymphocyte culture
PBL	:	peripheral blood lymphocytes
PBS	:	phosphate-buffered saline
PFU	:	plaque forming units
PI	:	post-inoculation
PHA	:	phytohaemagglutinin
S/C	:	subcutaneous
SI	:	stimulation index
U	:	International units
	:	blank in tables = not done
-	:	- in tables = result was negative

SUMMARY

The relationship between host and viral factors involved in Akabane viral infection of foetal lambs was investigated in the course of studies being made on immunogenesis in the foetal lamb. Emphasis was placed upon the development of foetal immunocompetence and its role in the pathogenesis of the disease. Viral pathogenesis was studied using embryological, virological, pathological and immunological techniques.

Embryonic implantation was found to be an extended process, commencing at 26 days gestation with the final foetal-maternal placental relationship being established by 40 days. While liver cells from young foetuses (38-59 days) gave only slight *in vitro* responses to mitogens and to alloantigens, they showed evidence of graft versus host disease after the injection of competent allogeneic lymphocytes. Thus foetuses of this age do not appear capable of an effective immune defence.

Adult sheep gave primary specific cellular and humoral immunological responses after infection with Akabane virus and then eliminated the virus without developing disease. Neonatal lambs gave similar specific primary humoral responses except for some slight delay in onset and eliminated the virus without developing disease. The neonatal lamb showed no cellular immune response.

Maternal infection was not demonstrated to cause infection of the pre-implantation embryo. Maternal infection

between 31 and 44 days resulted in infection of the foetuses and the development of lesions. Direct injection of the virus into the foetus between 50 and 126 days resulted in infection of the foetuses and lesions developed in those injected up to 120 days. Thus the virus caused lesions in the foetus when it was injected from the time of implantation until at least 120 days of gestation.

Akabane virus showed tropism for placental trophoblast and syncytium, central nervous system tissues, skeletal muscle and lymphocytes.

The nature of the lesions varied with age at infection with inflammatory infiltration occurring in all foetuses infected after 40 days but not in those infected earlier. The initial pathogenesis of the lesions appeared to involve direct necrosis of the infected cells by the virus. This damage was amplified by the mononuclear inflammatory cells where such an infiltration occurred.

Lymphocytes from uninfected foetuses of 71 days (the youngest tested) were capable of an adult-like response to mitogen. A lymphocyte response to Akabane virus, whether specific or non-specific, occurred within five days of direct infection of 50-120 day foetuses. Virus-specific cellular immune responses were just detected in a foetus of 94 days, and then from the earliest age at which serial testing was commenced, 108 days, specific sensitization of lymphocytes resembled that of adults.

Specific antibody against Akabane virus was first detected

at 75 days providing the foetus had been directly infected more than eight days previously. No specific antibody was detected by 75 days in foetuses infected at 31-44 days. The antibody appeared to be responsible for the elimination of viable virus from infected tissues.

Inactivated virus was much less effective than viable virus in inducing specific humoral or cellular responses.

No conclusive evidence was found for viral suppression of adult or foetal immunological function, although the absence of any detectable immunological response in foetuses infected before 41 days suggests possible impairment.

It appears from these experiments that natural Akabane viral infection of the foetus depends on the establishment of the placenta and that thereafter the foetus is susceptible to infection. The virus causes lesions in the foetus but not in the post-natal lamb or adult, and the nature of this disease varies with the foetal age at infection. The foetal lymphocyte is capable of carrying and possibly replicating the virus. The virus persists in the foetus until approximately 90 days. A lymphocyte response to infection is present by 58 days, which appears to influence the pathogenesis, and a specific cellular immunological response by 94 days. A specific humoral immune response develops at 75 days, which appears to be responsible for eliminating the viable virus from all tissues except placenta. Infection of the young foetus may suppress development of immunocompetence.

INTRODUCTION

INTRODUCTION

I. ONTOGENY OF THE IMMUNE SYSTEM

Knowledge of the pattern of development of the mammalian immune system is mandatory if any understanding of the mechanism of recognition of self, and therefore of the function of the mature immune system, is to be obtained. Information about the pattern of development of immunocompetence over the period during which acquisition of self-tolerance is assumed to occur is clearly essential. The present study investigated the development of immunocompetence in foetal lambs and the influence of antigenic challenge on this process. The aim was to provide information about modification of immune maturation by continuous antigenic exposure from an early stage of development.

Foetal lambs between 18 days (d) of gestation and parturition (at Day 149 $\pm$ 1) were used as subjects. The normal foetus over this age range has a "virgin" immune system protected from exposure to foreign antigens. It is therefore possible to examine both specific and non-specific aspects of the immune response, in a manner not possible in adults in which background non-specific responses, possibly due to previous antigenic stimulation, can complicate the interpretation of non-specific responses to a new stimulus.

The sheep is ideal for examination of immunological maturation. In contrast to most other species, the foetus is isolated from maternal immunoglobulins, and therefore any response detected can be presumed to be foetal (Miller, 1966). Additionally, the gestation period (148 - 150 days) is of

sufficient duration to permit the possibility of more 'spread' of the events of early gestation than occurs in small laboratory animals.

Of the antigens available for use as probes for the study of the development of foetal immunocompetence, the emphasis in this study was placed on a living microorganism. The response of the foetus to a pathogenic virus such as Akabane which can gain access without laboratory intervention is likely to represent more accurately the contribution that progressive maturation of the immunological system can make to normal foetal development. In particular, it should give an indication of the relevance of immune responses in the protection of the foetus from teratogenic effects of microorganisms.

Any pathological changes produced by a microorganism are likely to represent the outcome of interaction between effects directly mediated by the microorganism and the host response, whether immunological or otherwise. In the case of the developing foetus, the resultant pathology also reflects the further interaction of these two factors with developmental processes. The impact of the virus on the developmental process may result in morphological and/or functional abnormalities of organ systems. Additionally, concurrent developmental processes may influence the effect of the microorganism or the host response to the infection. Such an influence could conceivably be mediated in several ways. The first is the rate and timing of the development of foetal immune responsiveness, and of non-immunological forms of

protection. A second relates to the differing vulnerability of foetal target organs at various stages of their development. The third way in which the influence may be mediated is any modification in placentation or in the pre-placental relationship between mother and foetus which may occur at different stages of gestation.

(a) Morphology of the lymphoid system in the foetal lamb

The morphogenesis of many of the components of the immune system has been documented for the foetal lamb. Of the haemopoietic organs, the liver is present by 17 days after conception, although lymphopoiesis has not been histologically detected in foetal sheep liver of any age (Al Salami, personal communication). A lymphoid thymus is present by 36-42 days (Jordan, 1976), and an organized spleen by 58 days (Asantila and Toivanen, 1976), although splenic lymphocytes are not present in significant numbers until 70-80 days (Asantila et al, 1976); Al Salami, personal communication). Lymphocytes have been first detected in circulating blood at 32-40 days (Sawyer, Moe and Osburn, 1978; Asantila et al, 1976; Osburn, 1981) although Al Salami did not observe peripheral blood lymphocytes in significant numbers until 60 days, that is some time after thymic lymphopoiesis had commenced. He also observed that lymph node lymphopoiesis commenced at about 60 days, although Silverstein and Prendergast (1970) had previously reported the presence of lymphocytes in lymph nodes at 45 days. Other circulating leukocytes appear later. Neutrophils have been seen inconsistently in 60 day foetuses (Sawyer et al, 1978). However,

these neutrophils were not fully differentiated and therefore are unlikely to have become fully functional until birth or shortly before. Upcott, Hebert and Robins (1972) reported that eosinophils appeared at 85 days and monocytes and basophils at 100 days, while Sawyer et al. (1978) first observed basophils at 112 days, and Silverstein et al. (1970) detected monocytes from 58 days.

The leukocyte count of heart blood has been reported to rise to a peak at approximately 130 days, followed by a decline at 140 days, reflecting similar changes in the lymphocyte population (Upcott et al, 1972). The numbers of lymphocytes have been reported to rise sharply from 85 days to comprise 78.6 per cent of the nucleated blood cells (4.32 x 10^3/cmm) by 130 days, but then to fall to 63.8 per cent (1.78 x 10^3/cmm) by 140 days - a significant fall in the mean absolute count. During this time, the lymphocyte:neutrophil ratio remained greater than unity. Neutrophil numbers have also been observed to increase sharply at 135 days (Sawyer et al, 1978).

(b) Function of the immune system of the foetal lamb

Immunological defences available against microbial agents can take a number of forms in adult animals. Those likely to operate against Akabane virus in mature sheep include specific antibody production, specific and non-specific lymphocyte cytotoxicity, interferon production, phagocytosis, and complement mediated lysis of virus and viral infected cells (McConnell, Munro and Waldmann, 1981). As the foetus attains full immunocompetence, it would be expected to acquire

the capacity to employ all of these modes of defence.

The foetus has two positive responses to viral infection, even while immunologically immature. It possesses several non-specific capacities from an early age, including those of phagocytosis (Al Salami, personal communication) and interferon production (Banatvala, Potter and Best, 1971). It is likely, however, that the foetus produces less interferon than does the adult and that it is less sensitive to its action (Siewers, John and Medearis, 1970). The second response consists of the foetus's considerable ability to repair and replace damaged cells (Mims, 1968).

(i) Cellular function

There has been considerable investigation of the functional immune capacity of foetal lambs. Some capacity to respond immunologically appears to be acquired during the second month of gestation, but the sheep embryo (i.e. younger than 34 days) has not been studied. Proliferative responses to mitogens have been used in a number of species as a guide to the immunological competence or activity of an individual, as the response may be altered by the presence of disease or immune deficiency or by previous exposure to antigens (Schechter, Handzel, Altman, Nir and Levin, 1977; Hirsch, Griffin, Johnson, Cooper, de Soriano, Roedenbeck, Vaisberg, 1984). In general, it appears that while normal immunocompetent individuals respond significantly to mitogens, suppression of part or all of the immune system may depress the response to some mitogens. For example, suppression of T cell immunity may depress the response of lymphocytes to Con

A. In the foetal lamb, proliferative responses to mitogens have been detected in liver cells from 38 days (PHA and Con A), in the thymus from 68 days (PHA) and 90 days (Con A), and in the spleen from 98 days (PHA and Con A) (Leino, 1978). Leino and Soppi (1979) found that splenic and thymic lymphocytes responded to mitogens by 78 days, and to foreign liver cells by 48 days. Non-specific proliferative responses to allogeneic cells have been reported from 47 days (liver) and specific proliferative responses, identified by restimulation following specific elimination of the responding cells, to allogeneic cells have been reported from 58 days (thymus) and 70 days (spleen) (Asantila et al, 1976). Thus ovine foetal lymphocytes are capable of specifically recognizing allogeneic and xenogeneic cells almost as soon as lymphocytes are present in the foetal thymus and spleen, and this capacity develops roughly concurrently with mitogenic responsiveness of lymphocytes from organs other than the liver.

Further evidence of foetal cellular immunocompetence has been provided by the work of Miyasaka and McCullagh (1982) in which injection of allogeneic cells into foetal lambs between 50 and 60 days failed to induce post natal tolerance to allografts or to allogeneic antigens. Provided the conditions of antigen administration were appropriate for the antigen to behave as a tolerogen, that is, to induce immunological tolerance, this result suggest the likelihood that the foetus of 50-60 days may be capable of responding to the allogeneic cells, and thus possess some degree of immunocompetence. Alternatively, since there was no evidence of specific immunity in these lambs, the immune development may

have reached a stage at which the alloantigens induced neither tolerance nor immunity.

The response of liver cells is interesting in that, although both PHA and Con A are usually said to activate T cells (Janossy and Greaves, 1971), foetal liver cells respond to these mitogens when the thymus is barely lymphoid, that is before it would be expected to exert any influence on T cell maturation.

The ovine foetus acquires the capacity to reject skin allografts at approximately 70 days of gestation (Silverstein, Prendergast and Kraner, 1964; Neiderhuber, Shermeta, Turcotte and Gicas, 1971). That is, *in vitro* mitogenic and efferent mixed lymphocyte culture (MLC) responses have been observed before appearance of *in vivo* effector responses. The efferent arm of this rejection response has been studied *in vitro* using the cell-mediated lympholysis assay (CML). Foetal lambs showed clear MLC responses from 70 days gestation, but no capacity for CML was demonstrable. The CML response at birth was approximately one-seventh of the magnitude of the adult response (Granberg and Hirvonen, 1980). Thus the foetal development of immunologically specific effector T cells as determined by their CML capacity does not coincide with its presumed *in vivo* counterpart namely allograft rejection. This may be because *in vivo* rejection is a more sensitive indicator, or because allograft rejection and CML represent different functions. CML may not in fact have relevance for allograft rejection. The above reports also demonstrate that the appearance of specific T cell recognition in the sheep

precedes in ontogeny the development of the destructive T cell repertoire.

Infection with Bluetongue virus at 60 days results seven to ten days later in a mononuclear infiltration which the authors (Enright and Osburn, 1979 and 1980) suggest is cytokine-mediated and non-specific, and by 80 days the foetal lamb can respond to Salmonella antigen with neutrophilia (Fahey, 1977).

(ii) Interferon and complement

Production of interferon by the foetal lamb does not appear to have been studied. The complement system is present in the foetus, but its components occur in lower concentrations than in the adult, and are probably not fully functional (Osburn, 1981). This is supported by the fact that while measurable amounts of complement factor 1 (C'1) appeared in the blood plasma of foetal sheep on and after 39 days (Colten, Silverstein, Borsos and Rapp, 1968), haemolytic complement activity of sera was not detected in foetuses until 123 days, and its rate of development did not appear to be affected by exposure to a mixture of non-replicating antigens (Rice and Silverstein, 1964).

(iii) Humoral function

Osburn (1977) did not detect significant numbers of F_c receptors on normal foetal lymphocytes until after birth.

The normal foetal lamb is capable of synthesizing immunoglobulin (Ig) by mid-gestation. Fahey and Brandon (1978) detected IgM in foetuses from 70 days and IgG from 79 days.

Both Ig classes were also detected at 63-86 days, after administration of non-replicating antigens, and the authors observed that the synthesis of IgG was not dependent on the use of adjuvant. In another study, β_2M macroglobulin was detected in unstimulated foetuses of 80 days, and β_2M, β_2A and fast and slow gamma globulins in stimulated foetuses of 90 days (Silverstein, Thorbecke, Kraner and Lukes, 1963a). The authors suggested that some of the gamma globulin formed by the foetal lamb may lack specific antibody function.

In the absence of stimulation with Freund's complete adjuvant (FCA) almost all of the early antibody produced was β_2M, and little 7S gamma globulin was formed. On the other hand, the results of Silverstein, Uhr, Kraner and Lukes (1963b) imply that FCA may have suppressed the specific antibody response to ØX 174 phage, possibly by non-specifically "distracting" the limited number of available competent cells. Despite the capacity for antibody production demonstrated in the unchallenged foetus, Ig levels are low ($\lesssim$0.22 mg/ml IgG) unless the foetus has been stimulated by introduced antigen (Sawyer et al, 1978).

Lymphocytes capable of binding antigen have been observed from the earliest time of examination (58 days) (Decker and Sercarz, 1974). Cells bearing surface immunoglobulin have been detected in blood at 52 days (Symons and Binns, 1975). The frequency of these cells was 0.3 per cent at 56 days, 15.1 per cent at 78-87 days and 2.1 per cent at 117 days after which there was a second increase in frequency. The rapid increase between 56 and 82 days coincided with the period when

foetal antibody production against several experimentally administered antigens has first been detected.

Specific antibody production by the foetal lamb has been demonstrated following challenge with various non-replicating antigens. Silverstein et al. (1970) found that ØX 174 phage induced antibody formation six days after its injection at 35 days, and that maturation of responsiveness to other antigens occurred over a range of times from then until after birth, with antibody appearing to ferritin at 56 days and to ovalbumen at 125 days. Similar observations in foetal lambs have been reported by Fahey (1974) and by Fahey and Morris (1978). They found that the probability of a foetus mounting a specific antibody response at a given age depended on the antigen used. However, these foetuses developed the capacity to synthesize antibodies to most of the antigens over a fairly short period (between 64 and 82 days gestation). Sherwin and Rowlands (1975) who also observed a "heirarchy" of responsiveness to antigens in mice suggested that it might be related to the degree of thymic-dependence of the response induced by the different antigens. On this basis, thymus-independent antigens could induce a response in a foetus too young to possess differentiated T cells or organized lymphoid tissue. In such a situation the stage of development of the interactions between T cells, B cells and macrophages may be a more important factor in the capacity to respond to different antigens than the maturation of any one cell population or the regulated acquisition of V-region genes. The relevance for the foetal lamb of any concept of immunological maturation based on thymus dependence is questionable given the unimpressive

consequences of thymectomy in this species (Cole and Morris, 1971).

Very little has been done to investigate the capacity of ovine foetuses to respond to replicating antigens. Specific antibodies to several infectious agents have been observed - to Akabane virus at 65 days, 30 days after infection (Hashiguchi, Nanba and Kumagai, 1979), to Bluetongue virus at 95 days, 20 days after infection (Enright et al, 1980), and to Brucella ovis at 91 days, 11 days after infection (Osburn and Hoskins, 1969).

II. VIRAL INFECTION OF THE FOETUS

Viral infection of the foetal animal may have no detectable effect on the host, or it may result in morphological and/or functional abnormalities. There have been a number of investigations of mechanisms of viral pathogenesis relevant to congenital infection, particularly of the sheep.

(a) Preferential susceptibility of the foetus

A number of viruses cause disease of the foetus but not of the adult. These include Akabane virus (Parsonson, Della-Porta and Snowdon, 1977), the vaccine strain of Bluetongue virus (BTV) (Young and Cordy, 1964; Osburn, Silverstein, Prendergast, Johnson and Parshall, 1971b) and the vaccine strain of hog cholera virus (Young, Kitchell, Leudke and Sautter, 1955; Emerson and Delez, 1965). Other viruses produce foetal damage of greater severity and different type from that characteristic of the adult host. Examples include Rubella, bovine viral diarrhoea virus/mucosal disease and

feline panleukopaenia (Singer, Rudolph, Rosenberg, Rawls and Boniuk, 1967; Barlow and Gardiner, 1969; Margolis and Kilham, 1970).

Fucillo and Sever (1973) have proposed some reasons for the apparently greater susceptibility of the foetus to certain viruses:

(1) their state of immunological immunocompetence permits viral dissemination,

(2) rapidly dividing cells such as those found in the developing foetus are more favourable for supporting maintenance and replication of viruses, and

(3) foetal tissues produce less interferon than adult tissues, thus non-specific defences are less effective.

Mims (1968) considered that the embryo is very resistant to teratogenesis until the blastula stage, with earlier damage leading either to death or to recovery without malformation. The embryo becomes sensitive to teratogens when the primary germ layers have been laid down, and during organogenesis. After the organ systems have been defined they become less susceptible, so that subsequent damage is more likely to result in pathological lesions rather than malformations.

(b) Viral entry

In most congenital viral infections studied, foetal infection was preceded by maternal viraemia. Mims (1968), in reviewing the possible routes of infection of the foetus, considered the most important to be the chorio-allantoic placenta.

He summarized possible mechanisms of viral transport across the placenta as follows:

(1) transmission of virus particles from maternal to foetal circulation,

(2) passage of infected cells (platelets, blood cells) from maternal to foetal circulation, and

(3) establishment of an infectious focus in the placenta from which the virus can spread into the foetus.

(c) Target tissue damage

A number of possible mechanisms of tissue damage and/or malformation in foetuses infected with viruses have been suggested:

(1) placental vasculitis, with interference in placental function, e.g. Rubella (Tondury and Smith, 1966);

(2) virus-induced chromosomal injury. For example, Plotkin, Boué and Boué (1965) showed that Rubella will damage chromosomes of host cells in vitro, and Nusbacher, Hirschhorn and Cooper (1967), reported an increase in the average incidence of chromosome breaks in peripheral blood lymphocytes from Rubella-infected infants;

(3) interference with growth or maturation of tissues, as for example by inhibition of cell division or function. Hog cholera virus is considered to produce a non-cytolytic infection in which the malformations are caused by inhibition of cell division and function (Johnson and Byington, 1972). Rubella decreases the rate of cell replication (Plotkin and Vaheri, 1967). In Border disease of foetal lambs

caused by BVDV/MD, the lesions of the CNS reflect viral interference with normal myelination (Barlow and Storey, 1977);

(4) virus-induced cellular necrosis, as occurs with Herpesvirus (Horstmann, 1969), bluetongue virus (Osburn, Johnson, Silverstein, Prendergast, Jochim and Levy, 1971a), and feline panleukopaenia virus (Kilham, Margolis and Colby, 1967);

(5) interference with survival or growth of cells secondary to a disturbance of homeostasis. Hyperthermia in the last two-thirds of gestation has resulted in cerebral cavitation in foetal lambs (Hartley, Alexander and Edwards, 1974).

(d) Influence of host age on pathogenesis

The age of the foetus is another factor influencing the damage produced by viral infection and therefore the abnormalities resulting, in type and severity. Osburn et al. (1971a and 1971b) found that infection of foetal lambs with BTV at 50 days of gestation resulted in hydranencephaly, infection at 75 days in porencephaly, and at 100 days in mild focal encephalitis without gross lesions. They suggested that this may have been due to a greater susceptibility of the immature cells, but Naryan and Johnson (1972) considered that similar results (i.e. different pathological lesions at different ages) with BTV in newborn mice were due to the selective involvement of the germinal zones of the CNS rather than solely to immaturity or mitotic activity at a particular age. Cell populations pass through their peaks of division and differentiation at different ages. Provided some cell types are

more susceptible than others to a virus, an infection before the period at which maximum division or differentiation occurs may be eliminated before it can cause abnormalities. Feline panleukopaenia virus selectively attacks the external germinal layer of the cerebellum during the active phase of histogenesis, resulting in congenital cerebellar hypoplasia in foetal kittens (Kilham et al, 1967). As differentiation of the external germinal layer occurs very late in gestation, only infection occurring at this time results in cerebellar hypoplasia. As an example to this general situation, it has been suggested that swine fever virus may persist in pig foetal cells in latent form until the susceptible period of development occurs, when their teratogenic effect is expressed (Morgan and Wrathall, 1977).

Another factor associated with the age of the foetus, and capable of affecting the pathogenicity or apparent virulence of a virus, is the immunological status of the foetal host. Once any degree of immunocompetence has developed, immunological modulation of pathogenesis may be apparent in at least four ways:

(1) it may prevent or terminate a disease process, as in the case of BTV in sheep (Osburn et al, 1971a);

(2) it may induce damage in situations where the virus would otherwise have been inocuous, as is the case with lymphochoriomeningitis virus in mice (Hotchin and Weigand, 1961; Mims and Tosolini, 1969);

(3) it may alter the fundamental features of the disease, as it does in human Rubella (Singer et al, 1967) and ovine brucellosis in late gestation (Osburn and

Kennedy, 1966; Osburn, 1968). In both of these situations the foetus's newly competent immune system appears to contribute to the lesions which are apparent only in response to infection in late gestation;

(4) the virus may impair the development of the immune response. Togaviruses, in particular, appear to do this, with hog cholera, Rubella and BVDV/MD all capable of persisting after birth in animals infected during gestation (Johnson et al, 1972; Rawls, 1974; Terpstra, 1981). Persistence of BVDV/MD and hog cholera is often accompanied by a lack of detectable specific humoral response possibly due to virus-induced suppression of plasma cell development and impaired immunoglobulin synthesis (Terpstra, 1981). Persistence of Rubella virus in human babies is often accompanied by dysgammaglobulinaemia with IgM excess (Plotkin, Klaus and Whitely, 1966) and impaired cell-mediated in vitro responses (White, Leikin, Villavicencio, Abernathy, Avery and Sever, 1968).

Some of the mechanisms by which the immune system can produce host tissue damage while in the process of resisting viruses are known. Berger and Blanden (1981) reviewed the role of the T lymphocyte, and concluded that there were four ways in which it might be involved in producing host damage. These were cell-mediated cytotoxicity, virus-specific delayed hypersensitivity reaction, release of lymphotoxic factors, and regulation of other aspects of the immune response. These authors considered that the T lymphocyte plays an

essential role in the induction and amplification of inflammation.

Specific antibody in association with the virus may increase virus-induced tissue damage by enhancing virus growth in macrophages (Peiris and Porterfield, 1979) and thereby facilitating spread and amplification of the infection. Specific antibody may also be involved in hypersensitivity reactions. Of these antibody-mediated hypersensitivities, reviewed by McConnell et al. (1981), Type 1 (degranulation of mast cells or basophils) is unlikely in view of the lack of any reports of detection of IgE in ovine foetuses. Type 2 reactions in which antibody reacts with cell-surface viral antigen, resulting in lysis of the cell, would be possible, as would Type 3 reactions, where immune complexes form and in association with complement and accessory cells, produce tissue damage (necrosis and haemorrhagic vasculitis).

Of the viruses other than Akabane, which cause congenital infections of sheep, the most relevant to this study is BVDV (Border disease). Extensive investigation of Border disease of foetal lambs has raised the probability that host age influences the pathogenesis of this infection. Infection of pregnant ewes at 50-54 days results in infection of the foetal CNS, where the virus usually appears to be non-cytotoxic and to cause no inflammatory reaction. The main pathological change is interference with myelination of the CNS, resulting in congenital dysmyelinogenesis (Barlow and Storey, 1977). Precolostral sera from these lambs do not contain detectable specific neutralizing antibody (Vantsis et al, 1979) and they continue to excrete virus.

Barlow (1980) found hydranencephaly and cerebellar dysplasia due to liquefactive necrosis accompanied by cellular inflammation and perivascular cuffing in a proportion of the foetuses from ewes inoculated at 50-54 days; the damage was well established by 75 days. Most of these foetuses, unlike those showing only hypomyelinogenesis, had specific antibody at birth. Similarly, Clarke and Osburn (1978) inoculated 75 day foetuses directly with the BVD virus and found that this resulted in a disseminated encephalomyelitis, the most active inflammatory phase of which took place 12-14 days post inoculation, and which had substantially subsided by full term. The pathogenesis was thought to entail a destructive inflammatory process with reparative gliosis in the survivors and a resultant range of intracranial malformations and abnormalities.

Examination at birth of a group of foetuses from ewes infected between day 50 and day 130, revealed hypomyelinogenesis, milder in those infected after 70 days, and also periarteritis in those infected after 80 days (Barlow and Patterson, 1982). Barlow and Patterson (1982) concluded that either deviant differentiation producing hypomyelinogenesis, or necrosis with inflammation, may follow infection and may occur independently or in combination. A number of factors appeared to influence the type of pathological process which occurred. A dose-response relationship between the inoculum administered to pregnant ewes and the magnitude of the effect in newborn lambs was found by Barlow (1980). Both the strain of virus and the breed of sheep appeared to affect the type of malformations seen (Barlow, Vantsis, Gardiner, Rennie, Herring and Scott,

1980; Barlow, 1980).

The immune status of the foetus also appeared to influence the outcome of the infection. Barlow (1983) considered that immunological responsiveness to Border disease viral antigen developed at 70-90 days. Infection before this age induced a failure to produce specific antibody, which was interpreted as specific tolerance. Infection after 70 days induced a foetal inflammatory and humoral immune response which altered the type of abnormality observed. The reason for the occurrence of this secondary type of response in some of the foetuses from ewes inoculated at 50-54 days is not clear. Barlow interpreted this as a manifestation of "precocious" maturation.

(e) Influence of route of infection on pathogenesis

The effect of the route of infection on outcome does not appear to have been adequately investigated. It is possible that the inflammatory response observed by Clarke et al. (1978) was due to their direct inoculation of the foetuses. There is also a suggestion, from work with Border disease (Barlow et al, 1982) and Akabane disease (Kirkland, personal communication) that the outcome of the infection may be influenced in some undefined manner by the specific immune status of the mother.

III. VIRAL INFLUENCE ON HOST IMMUNE RESPONSE

In addition to producing tissue damage, some viruses may affect the subsequent immune responsiveness of the host by producing either non-specific depression, as in the case of

African swine fever (Wardley, 1982), or specific depression, or tolerance. Immunological tolerance is the specific failure of the immune system to respond to secondary challenge with an antigen, and is an acquired characteristic (McConnell et al, 1981).

The normal animal is assumed to have become tolerant of its own tissues (self-tolerance) before immunocompetence is attained. Additionally, the phenomenon of artificially induced tolerance of non-self antigens may occur under certain circumstances. The natural occurrence of tolerance of major histocompatibility gene-coded alloantigens was observed in chimaeric twin calves (Owen, 1945; Anderson, Billingham, Lampkin and Medawar, 1951), and was reproduced experimentally by the inoculation of embryonic and neonatal laboratory animals with foreign homologous cells (Billingham, Brent and Medawar, 1953, 1955). It was concluded that exposure of immature lymphoid tissue to antigen could result in tolerance rather than immunity to that antigen, and also that such tolerance was due to a central failure of the mechanism of reaction rather than to interference with peripheral effector mechanisms.

Specific tolerance has also been experimentally induced to non-replicating protein antigens. Examples include tolerance of bovine and human serum albumin in immature rabbits (Hanan and Oyama, 1954; Cinader and Dubert, 1955), killed Salmonella in chickens (Buxton, 1954), and Trichomonas antigen in calves (Kerr and Robertson, 1954). However, the results of attempts to induce tolerance of replicating antigens are

neither as frequent nor as clear cut. Tolerance could not be induced in the chick embryo by injection of bovine influenza virus into the yolk-sac or allantois (Burnet, Stone and Edney, 1950). A number of viruses persist with high levels of cell-free virus and deficient immune response in infected hosts. These include LCM (Traub, 1939)and various togaviruses including Rubella and BVDV/MD (Kennick et al, 1968; Westbury et al, 1979). On the basis of the viraemia observed in these conditions, tolerance has been suggested as a possible mechanism for this persistence. However doubt has been thrown on the central and specific nature of the observed immune failure in the case of LCM (Oldstone and Dixon, 1969; Silverstein, 1972); and Rubella (Michaels, 1969; Rawls, 1974), while in BVDV/MD persistent viraemia may be accompanied by the production of specific neutralizing antibody (Terpstra, 1981).

IV. AKABANE VIRUS

Although the immunological maturation of the foetal lamb has been investigated, the nature of the immune response to antigenic determinants carried by a virus to which the foetal lamb is likely to be exposed in normal husbandry has not yet been examined. In view of the difficulty encountered in earlier experiments and briefly described in Chapter Two, in finding an antigen which was not lethal after injection into the young foetus in utero, this study required a relatively non-pathogenic virus which could be injected into the mother and transmitted to the foetus, to produce a detectable but non-lethal infection. Two of the viruses endemic in the Australian sheep population satisfied these criteria, namely

Akabane virus, responsible for Akabane disease, and bovine virus diarrhoea/mucosal disease virus (BVDV/MD) which results in Border disease in sheep.

Akabane virus was selected for use for several reasons. Firstly, in natural infection, the virus can enter the foetal lamb at that stage of development when the animal is learning to discriminate self from non-self. This feature has major implications for investigating the discriminatory mechanisms responsible for determining whether the lamb's response takes the form of immunological tolerance or of immunity. Secondly, Akabane virus is a vector-borne virus. The Canberra district, from which the experimental sheep were derived and in which they were maintained during the experiments, is free of endemic Akabane virus infection; nor have epizootics been reported there. Thus the unexposed sheep necessary for such a study were readily available, and during experiments, sheep infected by different routes could be housed in the same insect-proof rooms without the possibility of cross-infection occurring to confuse the issue. BVDV, transmitted horizontally by contact between animals rather than by insect vector (Barlow et al, 1980) does not have such predictable geographic/climatic boundaries to its distribution, and the supply and maintenance of an extensively managed unexposed flock would be less certain.

The third reason is that a substantial body of information was available on the impact of Australian strains of Akabane virus on foetal sheep, and this background knowledge of pathology and virus strain pathogenicity was a major

advantage for the use of the virus as an antigen to examine foetal immunity.

A fourth reason for selection of Akabane virus was that BVDV has been reported to persist in sheep following foetal infection (Terpstra, 1981). This suggests that the foetal and neonatal immune response to this infection is abnormal, and while study of such an effect would be fascinating, it seemed less useful as a probe for the normal development of the immune system.

(a) Description

Akabane virus is a member of the Simbu group, genus Bunyavirus, family Bunyaviridae. Its main characteristics are as follows. The virions are spherical enveloped particles, 90-100 nm in diameter. The genome consists of single-stranded RNA, with a total molecular weight of 6-7 x 10^6, in three pieces. Virions develop in the cytoplasm and mature by budding into smooth-surfaced vesicles in the Golgi region or nearby (International Committee on Taxonomy of Viruses, 1982). Its electron microscopic appearance in suckling mouse brain and cell culture has been described by Ito, Kurogi, Takahshi, Goto, Inaba, and Omori (1979), who observed that particles obtained from both sources were identical, and were difficult to detect in both tissues.

(b) Epidemiology

The epidemiology of the infection in Australia has been extensively studied in recent years. Akabane virus infects cattle, sheep and goats. The infection is naturally trans-

mitted by a vector which is thought to be the blood-feeding midge Culicoides brevitarsis, as the virus has been simultaneously isolated from sentinel cattle and from nearby C. brevitarsis (St. George, Standfast and Cybinski, 1978). The infection is enzootic in northern Australia (Doherty, 1972; Della-Porta, Murray and Cybinski, 1976; Cybinski, St. George and Paull, 1978), and spreads south to produce epizootics in New South Wales when weather conditions are favourable for the transport and survival of the vector beyond its normal distribution (Hartley, Wanner, Della-Porta and Snowdon, 1975; Della-Porta et al, 1976). These epizootics appear to follow the river valleys, for example the Yass (Adamson, personal communication), Hunter and Nepean rivers and the south coast (Hartley, De Saram, Della-Porta, Snowdon and Shepherd, 1977).

(c) Clinical significance

Akabane infection of pregnant herds and flocks produces a significant disease. Akabane disease is characterized by the development of congenital lesions, especially arthrogryposis and hydranencephaly, in the progeny of susceptible animals. These lesions are often associated with abortion, dystocia and perinatal death. In contrast with these effects on the foetus, no clinical signs have been reported in adults or lambs infected postnatally, nor in foetuses of immune dams.

For nearly 40 years, there have been clinical outbreaks of arthrogryposis and/or hydranencephaly in sheep and cattle in New South Wales, for which an infectious cause was suspected but not identified (Blood, 1956; Hartley and Haughey, 1974a; Hartley and Wanner, 1974b). In some of the more

recent outbreaks this syndrome correlated closely with the presence of maternal and pre-colostral anti-Akabane antibody (Hartley et al, 1975). Consequently Akabane virus appears likely to be the cause of many of the early outbreaks.

Such epizootics have had a significant effect on herd and flock reproduction, especially in areas with concentrated lambing/calving seasons that are subject to outbreaks every three or four years. One such outbreak of congenital arthrogryposis and hydranencephaly probably due to Akabane virus, occurred in the Moss Vale district of New South Wales in the spring of 1964 and resulted in a loss of 5.4 per cent of the calves born (McClure and Dowell, 1968). Additionally, loss of cows as a result of dystocia has the consequence that even in commercial herds and flocks with an extended breeding season, the disease has major significance.

Fertility of the affected herds was lowered (McClure et al, 1968) as the first service non-return rate of the affected herds in both 1963 and 1964 was significantly below that of unaffected herds. This association of subnormal fertility with Akabane-induced effects strongly suggests that host factors may influence the outcome of exposure of a non-immune population to the virus. For example, the nutritional status of exposed animals, which commonly affects fertility, may also influence the course of Akabane virus infection and/or disease.

(d) Adult infection

Experimental infection of adult sheep with Akabane virus has been shown to result in viraemia without clinical disease

(Parsonson et al, 1977; Hashiguchi et al, 1979; Parsonson, Della-Porta, O'Halloran, Snowdon, Fahey and Standfast, 1981a). Viraemia was first detected one to eight days after intravenous inoculation of adult sheep and lasted for one to five days (Parsonson et al, 1981a). The only attempt to detect subclinical disease in animals infected post-natally has been that of Parsonson, Della-Porta, Snowdon and O'Halloran (1981c and 1981d). These workers examined semen from infected bulls, and also the genital tract of cows inoculated by intravenous or intrauterine routes at the time of insemination. The only abnormality detected was cellular infiltration of the corpus luteum.

Appearance of specific serum antibody is the only reported response of adult animals. This occurred within five to ten days of inoculation with three strains of Akabane virus (CSIRO 16, OBE-1, B8935) over a wide range of gestational ages (Parsonson et al, 1977; Hashiguchi et al, 1979). Parsonson et al. (1977) observed two peaks of antibody at ten days and 48 days after inoculation.

(e) Foetal infection

Although more work has been done on experimental infection of the foetal lamb with Akabane virus, these studies involved different strains of the virus, and were limited with respect to foetal age at the times of infection and at examination.

Three strains of Akabane virus have been used experimentally in sheep. Strain OBE-1 is a Japanese strain with which infection of foetal lambs has been attempted between

the ages of 29 and 101 days (Hashiguchi et al, 1979). Strain B8935 is an Australian strain which has been used to attempt to infect foetuses between 28 and 82 days gestation (Parsonson et al, 1977). Strain CSIRO 16 was also isolated in Australia, and foetal infection with this strain has been attempted between the ages of 32 and 36 days (Parsonson et al, 1981a). Series of experiments using different strains cannot necessarily be treated as identical.

(i) Viral entry

Foetal infection appears to be transplacental, rather than transovarial or vaginal, following maternal infection (Narita, Inui and Hashiguchi, 1979; Parsonson et al, 1981d). The stage of gestation at which the foetus is exposed seems to be of considerable importance in determining whether the virus will produce malformed foetuses (Parsonson et al, 1975, 1977). This may be because transplacental transmission of the virus is affected by the stage of pregnancy. Alternatively, the stage of foetal development might influence the infectivity and/or pathogenicity of the virus. Parsonson et al. (1977) infected ewes with the B8935 strain between 28 and 82 days gestation but could find evidence of transplacental transmission with foetal disease only in ewes infected between days 30 and 36. Transplacental transmission without disease was observed in ewes infected at 50 days. Thus transplacental transmission may occur only over a limited gestational range. In another study, Parsonson et al. (1981a and 1981b) used the CSIRO 16 strain to infect ewes at day 32 to 36, and found evidence of transplacental transmission and

disease in 40 of 55 foetuses. This report did not give information on whether virus transmission could occur without concurrent disease.

Hashiguchi et al. (1979) observed that transplacental transmission of the OBE-1 strain of virus occurred in ewes infected at 29-101 days. Gross pathological changes were evident in foetuses of ewes infected over the period of 29 to 69 days. However, results of histological examination of macroscopically normal lambs within this series were not included. Narita et al. (1979) reported disease in foetuses of ewes infected at 29-81 days but did not attempt to correlate this with evidence of transplacental transmission of the virus.

Thus, transplacental transmission appears to be possible in sheep infected by the intravenous route between days 29 and 101. Foetal infection outside this age range has not been reported. However, macroscopic and/or microscopic Akabane disease has only been observed in foetuses from sheep infected between 29 and 81 days. That is, there appear to be time limits within which viral transmission to the foetus can occur, and different time limits within which such infection produces foetal disease. However, these limits may vary with the virus strain involved, and there has been no consistent investigation of the foetal ages at which any one strain can result in infection and/or abnormalities of the foetus. In addition, examination of the effect of a range of doses of virus would be necessary to determine whether the time limits for infection or disease are as clear cut as they appear. This is unlikely to be possible with such an extended experiment in a

large species.

Furthermore, no studies of maternal haematogenous infection either very late in gestation or earlier than 28 days, (before embryonic implantation) have been reported, although such studies could well improve current understanding of the role of the placenta in viral pathogenesis. One study in cattle which examined the effect of exposure of the bovine foetus by intra-uterine injection of Akabane up to the early blastocyst stage did not result in foetal infection or disease, although some foetuses may have been lost (Parsonson et al, 1981d).

A strong indication of the importance of variations in placentation for transmission of the virus has been provided by Andersen and Campbell (1978). They found that whereas Akabane virus crossed the hamster placenta at most stages of gestation, it failed to cross the mouse placenta. Apart from the influence of transplacental transmission, the pathological changes produced in the foetus by Akabane virus are also likely to be related to its gestational age at the time of infection.

(ii) Pathological lesions

A progression of clinical syndromes has been observed in successively delivered calves during bovine epizootics. Polioencephalomyelitis precedes arthrogryposis, and hydranencephaly appears towards the end of the epizootic (Whittem, 1957; Hartley et al, 1977; Shepherd, Gee, Jessep, Timmins, Carroll and Bonner, 1978). This range of pathological changes is likely to reflect concurrent infection of foetuses

at a variety of gestational ages. The recent findings of Kirkland (personal communication) are in accord with this interpretation. He found during field observations of an Akabane disease epizootic in cattle (which have a gestation length of 282 $\pm$ 5.6 days) that calves born to cows infected between 76 and 104 days were more likely to manifest hydranencephaly, whilst arthrogryposis was commonest after infection between 104 and 173 days, and poliomyelitis was the principal feature following infection after 173 days.

Narita et al. (1979) infected pregnant ewes with strain OBE-1 at 29-36 days, 41-46 days and 81-91 days gestation, and found differences in the central nervous system (CNS) lesions of the foetuses and lambs from these three groups. They considered that the differences reflected the developmental stage attained by the foetus at the time of infection. This was in contrast with the earlier report of Parsonson et al. (1977), also working with sheep, who noted a wide variety of foetal pathological changes after infection of the dams within narrow time limits (30-36 days gestation). It was not apparent whether any correlation existed between the age at which any foetus was examined and the type or severity of its pathological features. It is possible that duration of foetal exposure to the virus, as distinct from age at the initial time of exposure, may also be an influential factor in the outcome.

The specific pathological features observed in lambs at 69-106 days gestation following maternal infection with CSIRO 16 at 32-36 days (Parsonson et al, 1981b) consisted of:

(1) central nervous system changes, namely hydranencephaly, porencephaly, cerebral cystic areas, malacia, oedema, subependymal gliosis, perivascular cuffing and mineralized plaques, of cerebrum, pons, cerebellum and spinal cord, hypoplasia of the spinal cord;

(2) locomotor system changes including skeletal muscle atrophy and degeneration, arthrogryposis, scoliosis and brachygnathism;

(3) pulmonary hypoplasia.

Similar changes were found in lambs at parturition following maternal infection with B8935 at 30-36 days, in addition to a decrease in the number of ventral horn motor neurones and in the quantity of myelin (Parsonson et al, 1977).

The abnormalities observed in foetal lambs 30 to 80 days after maternal inoculation with OBE-1 may be summarized according to the gestational age of the foetus at the time of inoculation.

(1) Lambs first exposed at 29-36 days displayed hydranencephaly, cerebral displacement, arthrogryposis, scoliosis, severe necrotizing meningo-encephalitis and swollen endothelial cells.

(2) Forty-one to 46 day old lambs showed multifocal encephalitis, porencephaly and ependymal rosettes.

(3) Inoculation at 81-89 days was associated with the occurrence of focal necrobiosis and gliosis in the white matter.

No spinal cord lesions were observed (Narita et al,

1979).

(iii) Virus distribution

From experiments using CSIRO 16 strain (Parsonson et al, 1981a and 1981b) and OBE-1 (Hashiguchi et al, 1979) it appears that, following infection of the ewe at 29-45 days of gestation, Akabane virus is recoverable from most foetuses 9-30 days later, most consistently from brain, placenta and allantoic fluid. However by 59-80 days after infection, i.e. after 100 days gestation, virus was only rarely recoverable and then only from the placenta.

(iv) Host immune response

As studies of Akabane viral infection have been aimed primarily at investigating the pathological features of the disease and the variation in virulence between strains of the virus, very little information has been collected on the immune response of either adult or foetal sheep to Akabane infection. Examination of the foetal immune response has been limited to testing for serum neutralizing antibody in infected foetuses and newborn pre-colostral lambs. Seventy-eight per cent of foetuses recovered at 76-105 days gestation from ewes infected at 32-36 days with CSIRO 16 were positive for specific antibody (Parsonson et al, 1981a). Thirty-nine lambs were born to ewes infected with B8935 at 28-82 days. Thirty-four of these had no lesions, and seven of the 39 had pre-colostral specific antibody (Parsonson et al, 1977). In a third study, Hashiguchi et al. (1979) infected ewes with the OBE-1 strain, and found specific antibody first in foetuses

of 64 days, 30 days after maternal infection, and more consistently in foetuses 75 days and older.

(v) Pathogenesis

Little is known of the details of pathogenesis of Akabane disease in the foetus. Neither the manner in which the various congenital abnormalities are produced nor the extent to which pathogenicity can be influenced by host factors is known. Describing the incidence and clinical findings of arthrogryposis and hydranencephaly outbreaks in calves, before the aetiological role of Akabane virus had been defined, Blood (1956) concluded that the arthrogryposis resulted from a neurogenic muscular atrophy following a degenerative neuropathy and Hartley et al. (1977) concurred with this view. An alternative conclusion was drawn by Kurogi, Inaba, Takahashi, Sato, Satoda, Goto, Omori and Matumoto (1977) to explain encephalomyelitis and polymyositis in an Akabane infected bovine foetus. While conceding that these changes could be sequelae of the CNS involvement, they considered that polymyositis resulting from infection of skeletal muscle cells seemed to be an important cause of the deformities and muscular damage observed in naturally affected foetuses and newborn calves. This interpretation was supported by the absence of spinal cord lesions, despite the presence of arthrogryposis (Narita et al, 1979). These authors also implicated direct destruction of undifferentiated neural cells, rather than secondary damage due to anoxia caused by vascular occlusion or haemorrhage, as essential in the pathogenesis of Akabane infection in the CNS.

(f) Assessment

The literature on Akabane virus infection of the foetus is a scattered group of studies using different Akabane strains and different animal species, with infection of the dam and recovery of the foetus being undertaken at a variety of ages. In many of the reports, virology, serology and pathology/histopathology were not correlated for individual foetuses. Consequently, comparison of results is often not feasible.

However, several suggestions about Akabane viral infection have gained some acceptance. The hypothesis that only infection in the first third of pregnancy produces foetal deformities (Kurogi et al, 1977; Parsonson et al, 1977) is not supported by all the literature. However, it does appear that viral pathogenesis is modified by the age of the foetus at the time of maternal infection, and that lesions may not develop if infection is delayed until late in gestation. The relative contributions of variations in stage of maturation of the placenta, with its altered transplacental transmission, the nature of the transmitted virus, the stage of development of the foetus, or duration of foetal exposure, remain unknown.

Secondly, despite the lack of information on subclinical disease, the absence of any clinical signs in infected adults suggests insusceptibility to damage by the virus. Since adult infection and humoral immune response have been demonstrated, this insusceptibility could be due either to efficacy of the immune response or to failure of the virus to

replicate in or to damage adult tissues.

Thirdly, the foetus may be capable of eliminating the virus after approximately 100 days from all tissues other than the placenta, although the defence mechanisms involved have not been determined.

The fourth point to have gained acceptance concerns the target tissues. Foetal brain and placenta appear to be important in the survival/replication of the virus, and brain is a major site of virus-induced lesions.

Finally, it appears that the adult produces specific neutralizing antibody in the usual manner of a primary humoral response, and that the foetus also develops this possible defence mechanism, in that it has the capacity to synthesize specific antibody, possibly as early as 64 days and certainly by 76 days.

Thus the areas of the subject of Akabane disease in the sheep which require further work are:

(1) the age range over which transplacental transmission can occur;

(2) the age range over which the foetus is susceptible to infection and disease;

(3) the possibility of occurrence of subclinical disease in the adult and the newborn and the pathogenesis of the foetal disease;

(4) the effect of the virus on the immunological responsiveness of adult and foetus.

The effect of infection upon the whole host organism (ewe and foetus) and the effect of the responding host upon the aetiological agent (Akabane virus) were studied conjointly. This method was advocated by Mims (1976) to investigate the pathogenesis of infectious disease. In the experiments of the current study, the primary aim was to examine the interplay, referred to at the beginning of the introduction, between the effects of the virus, the host response to that virus, and the developmental process in the foetal host. Within this aim, there were two areas of special interest. The first of these was the relevance of direct viral influences on the host response and of indirect influences such as the relative availability of the various types of response and the differing tissue susceptibility at different developmental stages. The second area of special interest was the relevance of viral impact on the host immune system, in particular the question of long-term alteration in subsequent immune responsiveness to the virus.

The first two chapters of this thesis describe the studies on implantation, placentation, and exposure of the early sheep foetus to allogeneic lymphocyte antigens. The remaining chapters describe the investigation of Akabane virus behaviour during infection of the sheep, and of the host response to this infection. The investigation was carried out under three categories: the response of adult ewes, the response of colostrum-deprived neonatal lambs, and the response of foetal lambs. The last category was further subdivided into three on the basis of the route of infection employed: transplacental, intravenous, and subcutaneous.

MATERIALS AND METHODS

MATERIALS AND METHODS

A. EXPERIMENTAL ANIMALS

1. Sheep: Mature (3 to 4-year-old) Merino ewes were purchased from the Canberra district which is usually free from Akabane virus infection, and grazed on improved pasture at the John Curtin School of Medical Research farm, Coppins Crossing, Canberra, A.C.T. The pasture diet of the sheep was supplemented with lucerne chaff and crushed oats (1:1 ratio) when necessary, and always when yarded and when housed during experiments when lucerne/oats became the sole feed. It was necessary to accustom ewes to eating lucerne and oats in the yards, otherwise they stopped eating altogether when brought indoors. Water was supplied to housed sheep in troughs and/or nipple systems. Housed sheep were kept in pens or in individual metabolism cages; in both cases, sheep were given at least five days to acclimatize before the experiment was commenced.

Each ewe was given one "Tri-sel" pellet (Arthur Websters, Northmead, Australia) to prevent any selenium deficiency which may have affected the viability of the embryo or foetus (Hartley, 1963). Periodically, blood samples were collected from these ewes and the sera tested for antibodies against Akabane virus (the technique of the plaque inhibition assay used is described below). All ewes remained seronegative before being brought into the laboratory. The ewes were joined with Merino rams (i.e. rams were placed in the paddocks with the ewes), which were fitted with Sire Sine harness and raddle (Hortico (Aust) Pty. Ltd., Victoria). The ewes were examined once daily for raddle marks as evidence of mating.

The day of marking was taken as Day 0 of pregnancy. Marked ewes were then placed with another ram fitted with a raddle of a different colour, to detect returns to service.

One group of ewes (those forming group 11) were treated to synchronize oestrus and induce multiple ovulation. An intravaginal progesterone sponge (Chronogest, Intervet, Australia) was inserted in each ewe and left in situ for 12-20 days; on the day preceding removal of the sponge the ewe received 1500 U. of PMSG (Follicon, Intervet International B.V., Boxmear, Holland) by intramuscular injection. On removal of the sponge, the ewe was yarded with the ram until marked, when the raddle was changed.

Ewes which held to service were divided into eleven groups.

Group 1. Ewes in this group were anaesthetized with sodium pentobarbitone between days 39 and 76. Thirteen foetuses were removed from 12 pregnant ewes. The foetuses were used in lymphocyte transformation assays and as controls in experiments described in Chapters 5 and 6.

Group 2. Eight ewes were anaesthetized between days 24 and 59 of gestation and their foetuses injected with allogeneic lymphocytes 2.5 h before they were removed for histology and lymphocyte assays.

A further five ewes were anaesthetized between days 29 and 50, and their foetuses inoculated with radioisotope-labelled allogeneic lymphocytes 2.5 h before autopsy, when foetal tissues were prepared for autoradiography.

Group 3. Three non-pregnant ewes were not treated, and were blood-sampled to provide controls for adult peripheral blood lymphocyte (PBL) *in vitro* reactivity.

Group 4. Ten ewes were inoculated intravenously (I/V) with Akabane virus, and PBL and/or efferent lymphocytes were taken at intervals and assayed for *in vitro* reactivity.

Group 5. Twenty-nine ewes were inoculated I/V with Akabane virus between 31 and 44 days, and their 32 foetuses removed at intervals until day 75.

Group 6. Ten ewes were anaesthetized between days 45 and 120 and their ten foetuses directly inoculated intraperitoneally (I/P) with Akabane virus. These foetuses were removed five to 15 days later.

Group 7. Ewes in this group were anaesthetized between days 104 and 135, and the prescapular efferent lymphatic ducts of the eight foetuses were cannulated. One foetus was left unchallenged. The local prescapular node of the remaining foetuses was challenged with Akabane virus or a Vero cell control inoculum.

Group 8. Ewes in this group were left to lamb normally. The four newborn lambs were used in the study of neonatal response to Akabane virus described in Chapter 4.

Group 9. The three ewes in this group were anaesthetized between days 24 and 29, when the pregnant uteri were perfused with carbon, fixed and removed for histological examination.

Group 10. These four ewes were anaesthetized at day 18,

and Akabane virus injected into the middle uterine artery supplying the pregnant horn. Five days later, they were again anaesthetized and the four embryos removed and fixed for electron microscopical examination.

Group 11. A majority of the ewes of this group had been treated to synchronize oestrus and to induce multiple ovulation, as it was an advantage in this experiment to obtain a number of embryos with similar genetic composition and environmental influences in order to have minimal uncontrolled variation. Nineteen pregnant ewes were anaesthetized between days 18 and 29. Their twenty-seven embryos were removed and placed in *in vitro* culture systems.

All sheep inoculated with virus were kept in insect-proof accommodation.

2. Rabbits: Adult male rabbits of the Belgian Lop-ear strain, bred by the Animal Breeding Establishment of the John Curtin School of Medical Research, were used for the production of antibody to Akabane virus. They were maintained in individual cages in an insect-proof room, and fed commercial rabbit pellets (Doust and Rabbidge Pty. Ltd., Concord West, Australia) *ad lib*.

B. SURGICAL TECHNIQUES

The ewes were brought in from pasture at least five days before surgery to accustom them to their new food, environment and handling.

1. Anaesthesia and pre-operative preparation: for 24 h before the operations, food was withheld, while water was

freely available. The animals were then anaesthetized with five per cent sodium thiopentone (Ceva Chemicals, Australia) given intravenously into the jugular vein to effect, i.e. until anaesthesia was sufficient to enable tracheal intubation (approximately 7 mg/kg bodyweight), and a Magill cuffed endotracheal tube (size 9) was introduced into the trachea. The ewes were placed on the operating table in dorsal or lateral recumbency, and anaesthesia maintained with one to two per cent halothane (ICI, Australia) in one to two L/min oxygen (CIG, Australia), the halothane being delivered by a Goldman Halothane Vapouriser (British Oxygen Co., London, England). The incision site was shorn of wool, shaven, scrubbed with Betadine (Faulding and Co., Thebarton, Australia), and disinfected with 0.5 per cent w/v hibitane (ICI (Aust), Villawood, Australia), in 70 per cent v/v alcohol. Surgery was performed aseptically.

2. Inoculation of the foetal lamb: A 12-15 cm ventral midline incision of the abdomen of the ewe gave access to the uterus, which was partially exteriorized and covered with damp swabs and/or encased in a plastic bag containing a small amount of warm saline. Access to the foetus was obtained through a transverse incision in the pregnant horn. This incision was made using electrocautery (Endotherm surgical unit, Allen and Hanburys, London), on the dorsal surface overlying the foetus, in an area containing a minimal number of blood vessels. The length of the incision varied with the route of injection - approximately two cm was sufficient to permit intraperitoneal inoculation, while intravenous injection required exteriorization of umbilical cord or foetus, and there-

fore a two to five cm incision.

Three foreign substances were injected into foetuses. The first of these, Akabane virus (2×10^6 PFU in 0.2 ml) was injected I/P, through a 26 gauge hypodermic needle passed through the window made in the uterine wall, through the foetal membranes and into the abdomen of the foetus which had been correctly positioned by 'ballottement'. This was because the exteriorization and return of the young foetus necessary for injection into the umbilical cord appeared to encourage foetal death, and therefore could not be used where survival of the foetus was required for more than several hours. However, when foetuses were to be labelled with radioactive isotope, the isotope (50 µCi of ^{3}H-thymidine in 0.2 ml Hanks' balanced salt solution (BSS)) was injected into the umbilical vein through a 26 or 30 gauge needle after the foetus was exteriorized, after which the foetus was wrapped in damp swabs until removal several hours later. Allogeneic cells (4.0×10^8 cells in 0.2 ml Hanks' BSS) were similarly injected into the umbilical vein, as foetuses were to be autopsied several hours later.

The uterine incision was closed with continuous inverting mattress sutures, using M 2.00 silicone-treated, braided silk (Davis and Geck, American Cyanamid Co., New York). The abdominal incision was closed in three layers with M 5.00 silk.

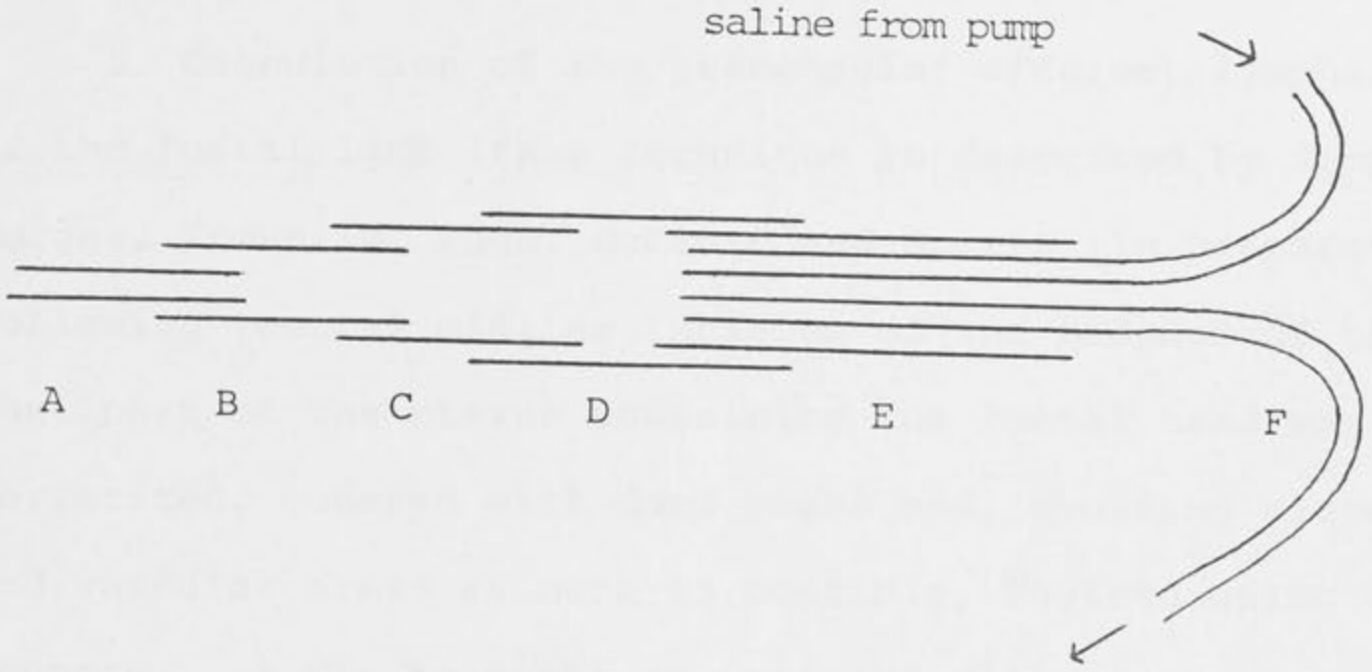
3. Recovery of the embryonic and foetal lamb: The embryo or foetus was exposed as for inoculation. Embryos (younger than 35 days) were flushed out with membranes intact by the injection of sterile saline into the cranial end of the preg-

nant horn. In the case of foetuses, the membranes were ruptured, the foetus exteriorized, and the umbilical cord ligated and cut. Before autopsy, foetuses were killed by section of the cervical cord.

At the end of the experiment, ewes which had been exposed to virus were then destroyed by sodium pentobarbitone overdose (Lethabarb, Arnolds of Reading, Australia). The uterus and abdomen of non-exposed ewes were closed as described above, and the ewes returned to the flock.

4. Cannulation of the popliteal lymphatic duct of the ewe: The procedure was based on that described by Hall and Morris (1962). With the ewe in lateral recumbency, a 12 cm incision was made in the skin distal to the greater trochanter, overlying and parallel to the caudal border of the biceps femoris muscle. By blunt dissection between biceps femoris and semimembranosus muscles, the efferent lymphatic duct was isolated, tied off with M 2.00 silk, and a polyvinyl cannula of internal diameter 0.58 mm or 0.8 mm (SV 45 or SV 55, Dural Plastics and Engineering, Australia) was inserted and tied in place. The distal end was passed out through muscle dorsal to the incision and held in place at the point of exit with a purse-string suture. A plastic bottle-holder sutured to the skin supported a 250 ml tissue-culture flask (Corning Ltd., Stone, England) containing approximately 2 mg of a mixture of 500 mg Streptomycin, 100,000 units Penicillin G and 100,000 units Heparin sodium (CSL., Melbourne). The distal end of the cannula was led into the flask through a hole in the lid. Any other efferent lymphatic ducts present were tied off. The fascia

Fig 0.1 Construction of cannula for foetal efferent lymphatic vessel.



	Internal-external diameter (mm)	Approximate length
A	0.2 - 0.5	6 cm
B	0.5 - 1.0	6 cm
C	1.0 - 2.0	1 cm
D	2.0 - 3.0	1 cm
E	1.5 - 2.5	1 cm
F	0.5 - 0.9	2 m

was closed with single interrupted sutures of M 4.00 silk, and the skin with Michel clips (Gebrüder Martin, Tuttlingen, F.R. Germany). In some cases, an afferent lymph duct was also cannulated in a similar manner, except that other afferent ducts were not tied off.

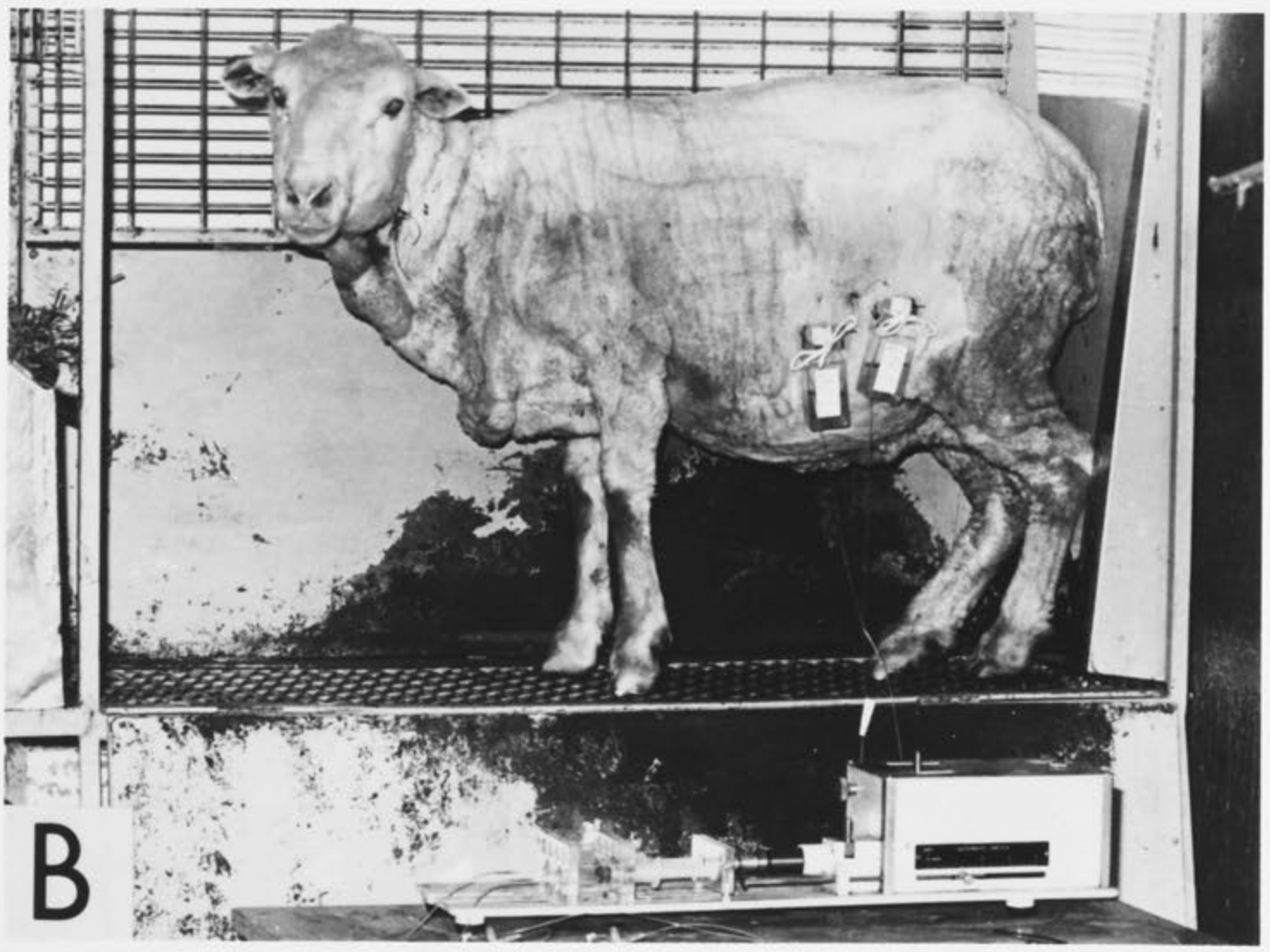
5. Cannulation of the prescapular efferent lymphatic duct of the foetal lamb (this technique is described by Simpson-Morgan, Trevella, Hugh, McClure and Morris (in preparation)): Following ventral midline incision of the abdomen of the ewe, that part of the uterus containing the foetal head was exteriorized, covered with damp swabs and, avoiding placentomes and vascular areas as much as possible, incised using electrocautery. A six to eight cm incision allowed exposure and return of the foetal head. The foetal membranes were incised and anchored to the uterine incision with artery forceps. Foetal fluid was removed by suction to a sterile flask containing 1.0×10^6 units Penicillin G, to be replaced when the membranes were closed. The head was exteriorized and wrapped in swabs kept damp with warm saline ($37^{\circ}C$). A five cm skin incision was made one cm lateral and parallel to the trachea, and the prescapular efferent lymphatic duct exposed by blunt dissection. Where several ducts existed, all but one were tied off, and the remaining efferent vessel cannulated with polyvinyl tubing of internal diameter 0.2 or 0.5 mm (SV 8 or SV 37), filled with 40 U/ml heparin in 0.9 per cent saline, and free from air bubbles (Fig. 0.1). The skin incision was closed with single interrupted sutures of M 3.00 silk, the cannula tied to the skin, and the lymph node challenged by subcutaneous injection of the inoculum in the area drained by

PLATE 0.1 Collection of lymph from newborn and foetal lambs.

A: The efferent cannula from the newborn lamb leads from its point of exit in the neck into a collection flask tied to a stocking vest. A second stocking protects cannula and flask.

B: The efferent cannulae from the foetus lead into collection flasks sutured to the abdominal wall of the ewe. Heparinized saline is pumped through the efferent cannulae by the slow infusion pump visible in front.


A


B

the prescapular node (i.e. cranial to the node). For some foetuses, the prescapular efferent lymphatic duct on the other side was similarly cannulated. The foetus was then replaced in the uterus, the foetal fluids replaced and the membranes closed by tying them together with M 3.00 silk. The uterine incision was closed by continuous inverting mattress sutures (M 3.00 silk), the two arms of the cannula being brought out through the suture line, and the uterus replaced. The cannulae were then threaded through one end of a sharpened brass rod 20 cm in length and 0.5 cm diameter, and taken out through the abdominal wall, where they were fixed by a purse-string suture and tied to the skin. Approximately 0.75 m of the cannulae was left inside the abdomen to prevent their rupture by parturition. The afferent arm of the cannula (see Fig. 0.1) was attached to a Palmer slow infusion pump (C.F. Palmer Ltd., London), pumping sterile Dulbecco's phosphate buffered saline to which had been added 80 U/ml heparin sodium (Heparin Injection BP, CSL., Melbourne) and 200 U/ml Penicillin G. The efferent arm was placed in a collection bottle which was tied to a bottle holder in turn tied to the skin (Plate 0.1). Abdominal closure was performed in three layers with M 5.00 silk. On recovery, the ewe was placed in a metabolism cage.

6. Cannulation of the foetal jugular vein: Following exposure of the foetal head, the skin was incised for three cm over and parallel to the jugular furrow, and the jugular vein exposed by blunt dissection and ligated (M 2.00 silk). An artery clip was placed proximal to the ligature to prevent blood loss while the vein was incised and a polyvinyl cannula

(SV 45), filled with heparin/saline, inserted. The clip was released, the cannula was adjusted, tied in place with two ligatures of M 2.00 silk, and taken out through the skin incision before being tied to the skin. Foetus and ewe were closed as described above.

Foetal blood samples of 1.5 ml were collected by suction into a fresh sterile syringe, after the heparin-saline had been withdrawn from the cannula. After sampling the cannula was refilled with heparin-saline and tied off.

7. Foetal lymphatic cannulae: These were constructed from polyvinyl tubing (Dural Plastics) as shown in Fig. 0.1. Cyclohexane was used to glue together the components, and all cannulae were gas sterilized (ethylene oxide) before use.

C. VIROLOGICAL AND SEROLOGICAL PROCEDURES

1. Source and preparation of Akabane virus: For the infection of animals of Group 5, i.e. maternal inoculation preceding transplacental transmission, the CSIRO 16 strain of Akabane virus was used after passage through mouse (M) and mosquito (MOS) as follows: Mx2/MOS/M/MOSx2/M. The source and early history of the virus were described by Parsonson et al (1981a) and St. George et al (1978). For infection of all other sheep the same virus was used, after a further passage through Vero cells in vitro. This passage was also used in all in vitro assays. Both virus preparations had titres of 1.0×10^7 PFU/ml. A concurrent Vero cell culture, not inoculated with virus, was harvested, exposed for five seconds to ultrasonic radiation from a Sonifer B-12 (Branson Sonic Power Co., Danbury, U.S.A.) and stored at -160^{o}C as was

the Akabane-infected culture, and later used in Groups 7 and 8 and in _in vitro_ assays as 'Vero control' for the Akabane virus.

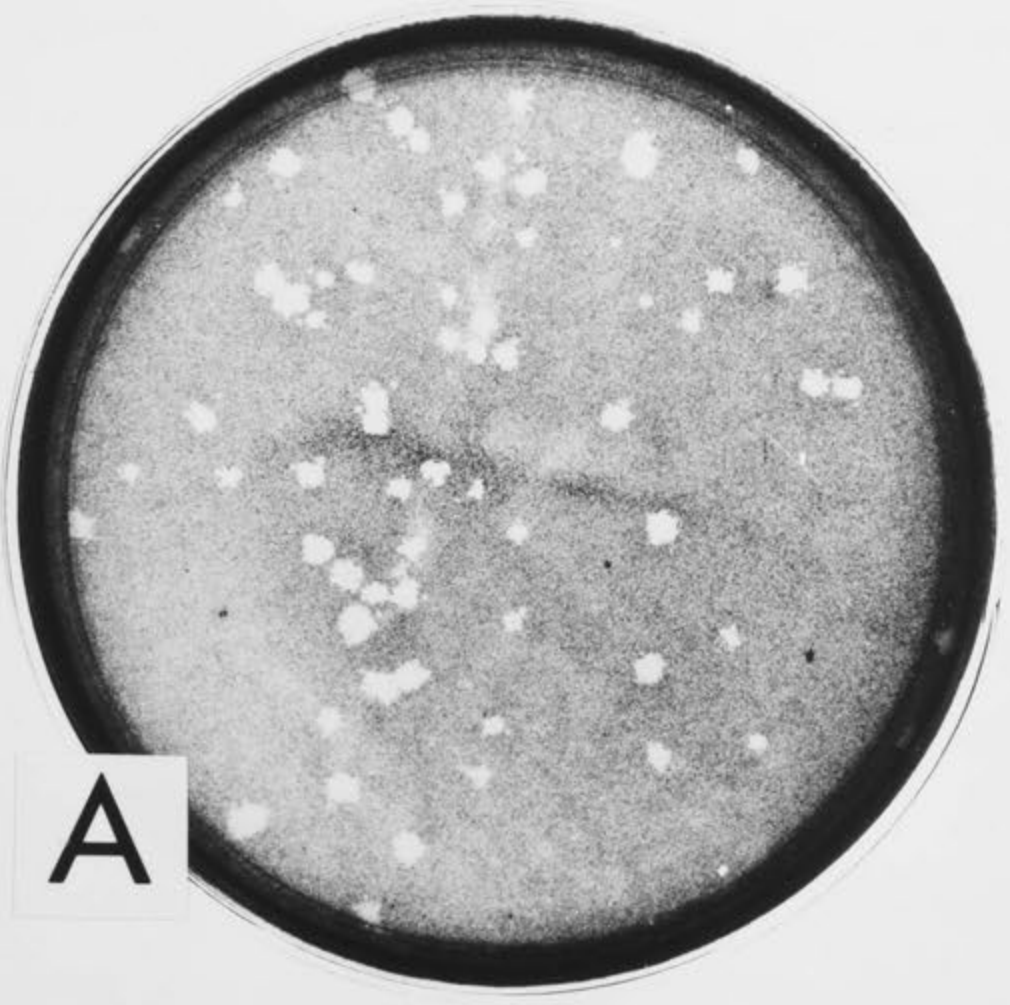
Where required, the virus was inactivated by ultra-violet irradiation from an Oliphant (Aust) Germicidal lamp, G15T8, two inches from the source, for 30 minutes at room temperature, followed by storage at -20°C (a technique adapted from that of Griffin and Johnson, 1973). Titration of the resulting preparation produced no plaques. Inactivation by exposure to β-propiolactone (βPL) was tried but the resulting preparation proved toxic to the lymphocytes _in vitro_ (see Fig. 0.3), and this method was abandoned. βPL inactivation followed the method of Della-Porta (pers. comm., 1982) and involved the addition of ten _per cent_ v/v of 1 M Tris-HCL (Sigma, St. Louis, U.S.A.), pH 8.0, followed by 0.2 _per cent_ βPL at 4°C for ten minutes, stirring every minute. The suspension was then placed at 37°C for two hours, stirring every 15 minutes, the pH adjusted if necessary, and the preparation stored at 4°C.

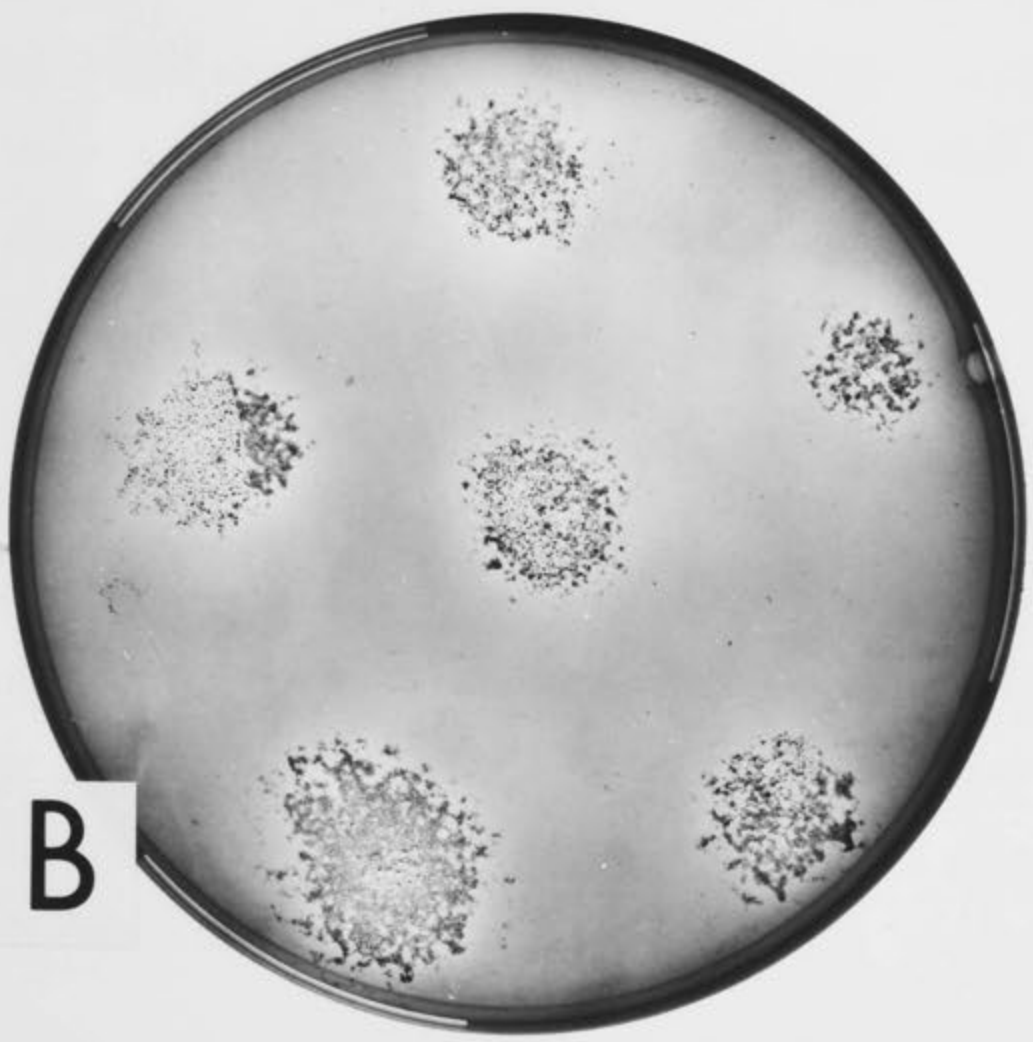
2. Plaque assay for titration of Akabane virus: This technique was modified from that of Kurogi _et al_ (1976), by the use of Vero cells instead of HmLu cells. Akabane virus was detected in tissues and titrated by means of the plaque assay. A sample (approximately 1.0g) of the tissue was weighed, then ground in a sterile pre-cooled mortar and pestle to a homogeneous slurry, using a small amount of sterile aluminium oxide number 90 grit. Hanks' balanced salt solution containing 0.2 _per cent_ bovine serum albumin (Hanks' BSA) was added making a final volume of ten _per cent_ w/v, and the homo-

PLATE 0.2 Vero cell monolayers after titrations of Akabane virus and neutralizing antibody.

A: Typical plaques of cell lysis after plaque assay of placentome from an Akabane-infected foetus for Akabane virus.

B: Typical zones of inhibition of cell lysis after plaque inhibition assay of lymph and sera from Akabane-infected sheep for specific neutralizing antibody to Akabane virus.

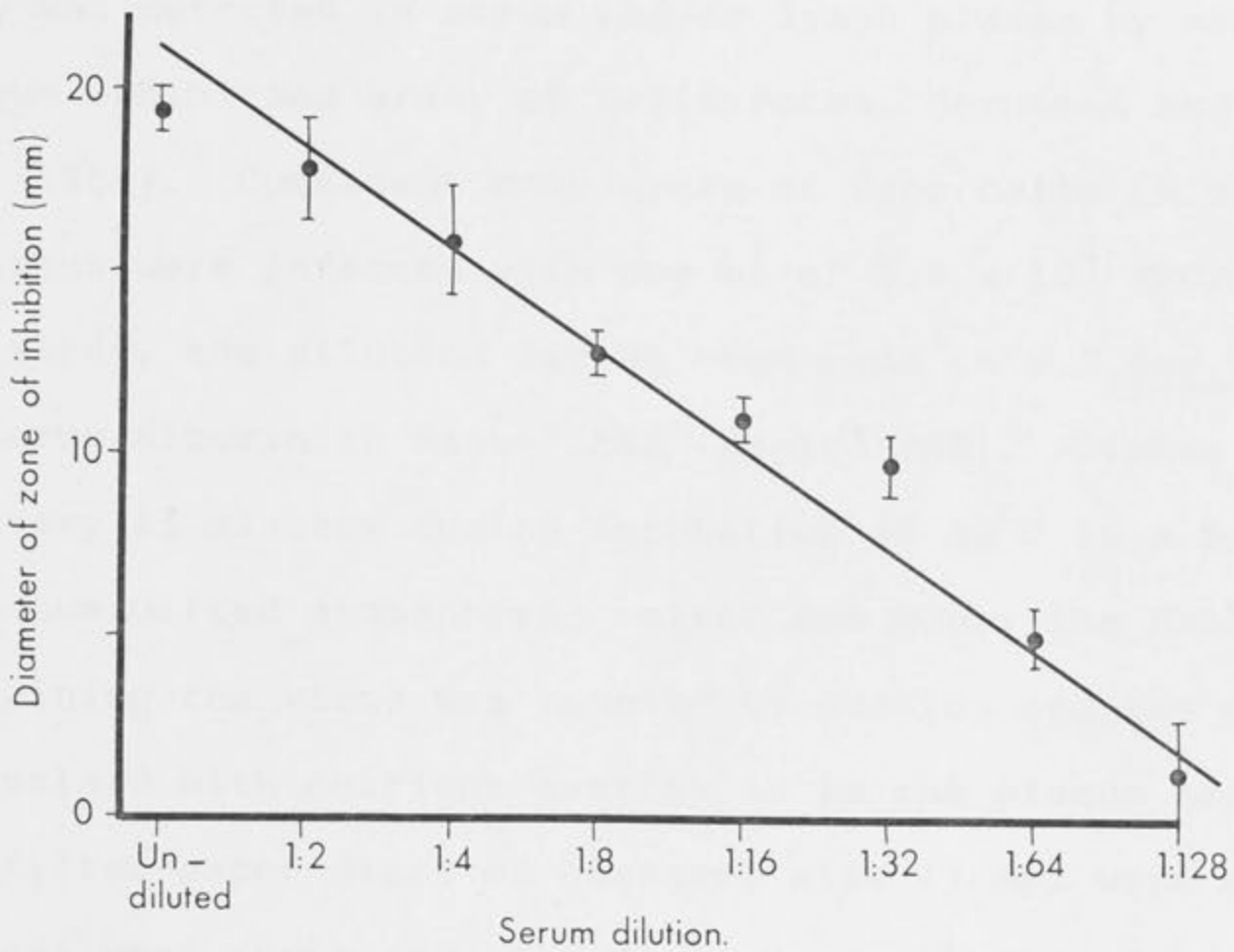

A


B

geneous suspension clarified at 10,000 G for ten minutes at 4°C. The supernatant was then immediately assayed. Tenfold dilutions in Hanks' BSA were prepared, and duplicate confluent monolayers of Vero cells (in 50 mm glass petri dishes) were inoculated with 0.2 ml of each dilution. Plates were rocked every 15 minutes during a one-hour incubation at 36°C in a five per cent CO_2 humidified atmosphere, after which the inoculum was removed by suction and five ml of nutrient overlay (1% Bacto agar (Difco Laboratories, Detroit, U.S.A.), 5% foetal calf serum (CSL., Melbourne, Australia), 0.05% DEAE-Dextran (Pharmacia, Uppsala, Sweden), 30 mM HEPES (Sigma, U.S.A.), 0.2% $NaHCO_3$, 100 U/ml Penicillin, 100 µg/ml Streptomycin, 100 µg/ml Neomycin, in Medium 199 (CSL), pH 7.4) were added to each plate. Following incubation in a humidified five per cent CO_2 atmosphere at 36°C for three days, the cultures were stained with Neutral Red (0.01% Neutral Red, 1% agar, in distilled water) and returned to the incubator for 12 hours. Non-stained plaques where the cells had lysed were counted, corrected for dilution, and the titre expressed as plaque forming units (PFU)/ml of the ten per cent suspension (Plate 0.2). Each assay included the titration of an Akabane virus suspension of known titre (as described in C.1.) as a control. The test sample was also inoculated into duplicate Vero monolayers which were incubated for one hour at 36°C. Five ml of maintenance medium (described below) were added to each plate, and the cells scored for cytopathic effect after three days further incubation at 36°C in five per cent CO_2.

Where foetal or adult bovine serum was used in in vitro procedures concerned with Akabane virus or antibody against

Fig 0.2 Effect of serum dilution on the diameter of the zone of inhibition in the plaque inhibition assay.



The values plotted are the mean values of inhibition of Akabane virus of four sera from adult ewes infected with Akabane virus four weeks previously.

Akabane virus, such serum was obtained from Victoria where the presence of Akabane virus has not been reported, and was assayed in the laboratory to confirm the absence of the virus and of the neutralizing antibody.

3. Plaque inhibition assay for detection of specific neutralizing antibody to Akabane virus: Specific neutralizing antibody was detected in serum and/or lymph plasma by means of the plaque inhibition assay of Della-Porta, Herniman and Sellers (1981). Confluent monolayers of Vero cells in 90 mm petri dishes were infected with one ml of 5.0×10^4 PFU/ml Akabane virus, the dilution having been made in 0.2 per cent bovine serum albumin in Hanks' BSS (Hanks' BSA). Dishes were rocked every 15 minutes during incubation at 36°C in a five per cent CO_2 humidified atmosphere; after one hour, the Hanks' BSA containing the virus was removed by suction and the monolayer overlaid with nutrient overlay as in the plaque assay. Sterile filter-paper discs of constant size (7 mm) were soaked in the undiluted serum and placed on the overlay surface (six discs/plate). Duplicate plates were prepared, and positive and negative control sera included. Plates were inverted and incubated for three days, when the discs were removed. The cultures were stained with Neutral Red, as in the plaque assay, and incubated for 24 hours. Zones of inhibition of cell lysis (Plate 0.2) revealed the presence of antibody, and measurement of the mean zone diameter was used to provide semiquantitative titres (Fig. 0.2).

4. Production of antibody to Akabane virus: Antibody to Akabane virus was raised in rabbits that were housed in insect-proof cages. Two ml of Hanks' BSA containing 1.0×10^7 PFU

of virus was emulsified in two ml Freund's complete adjuvant (Difco, U.S.A.), and 0.1 ml of the emulsion was inoculated at each of 40 sites over the clipped back of the rabbit. Three months later the rabbit was inoculated intramuscularly with 1.0×10^7 PFU of virus without adjuvant and this was repeated one month later. Rabbits were bled from ear veins once weekly following the third inoculation until the specific neutralizing antibody response was optimal, when they were bled out under sodium thiopentone anaesthesia. The serum was recovered after coagulation of the blood, centrifuged to remove red cells, and the pooled sera stored at $-20^{\circ}C$.

D. IMMUNOFLUORESCENT PROCEDURES

The procedures used were based on those described by Johnson, Holborrow and Dorling (1978) and Nairn (1976).

1. Fluorescent staining of Akabane viral antigen in foetal tissues: Duplicate cryosections of foetal tissues were fixed in acetone for ten minutes. One section was then covered for 30 minutes with undiluted rabbit anti-Akabane serum in a humidified atmosphere, while its control was covered with undiluted Akabane antibody-negative rabbit serum. The sections were then washed three times in phosphate-buffered saline (5 minutes each) before being covered with fluorescein isothiocyanate-coupled sheep anti-rabbit immunoglobulin (courtesy of Dr. Willenborg) for 30 minutes. After three further washes (total 5 minutes) the sections were examined under a Leitz Orthoplan microscope at x 400 magnification, illuminated for fluorescence by a 200 W Mercury lamp with a Leitz I_2 block (i.e. wavelength of exciting filter 450-490 nm, wavelength of suppression filter 515 nm) filter. Further sections of some

specimens were counterstained to reduce non-specific fluorescence, by staining in 0.1 per cent Evan's Blue for five seconds before the final washes. Each section was compared with its control, therefore only those sections showing specific fluorescence were scored as positive. Infected Vero cell monolayers were fixed and stained similarly as positive controls.

2. Fluorescent staining of surface immunoglobulins on foetal efferent lymphocytes: Foetal efferent lymphocytes were examined for the presence of surface-bound immunoglobulins. The cells were washed twice in PBS, resuspended in 0.2 ml PBS and stained for 30 minutes with a 1:4 dilution of fluorescein-conjugated rabbit anti-sheep Ig or IgM (Dakopatts, Denmark). The cells were then washed twice in PBS, resuspended to a concentration of 1.0×10^7 cells/ml and 400 counted under a Leitz Orthoplan microscope at x 400 magnification, illuminated for fluorescence by a 200 W Mercury lamp with a Leitz I_2 filter block. In each sample, the number of fluorescing cells was expressed as a percentage of the 400 cells counted.

E. MEASUREMENTS OF EFFERENT LYMPH

Efferent lymph was collected continuously from cannulated nodes, collection flasks being changed every 12 hours. Thus measurements were made on the lymph accumulated over the previous 12 hours.

1. Lymph flow rate: The flow rate was determined by measuring the volume of lymph at each 12-hourly collection and was expressed as ml/h. Where saline was pumped into the afferent arm of the efferent cannula, its flow rate was con-

stant and known, and the volume of saline delivered was deducted from the total volume after each collection.

2. Efferent cell counts: The total cell count and large cell count were determined for each collection using a Coulter Counter, Model Fn (Coulter Electronics, Dunstable, England) and are expressed as cells/h. A cytocentrifuge smear was made from alternate collections (i.e. 24-hourly) using a Cytospin (Shandon Elliot) cytocentrifuge, 1000 rpm for ten minutes, and stained with "Diff-Quik" modified Wrights stain (Harteco, AHS/Australia): differential counts were made from these to confirm the cell types being counted on the Coulter counter.

F. SEPARATION OF PERIPHERAL BLOOD LYMPHOCYTES

The technique of Fiscus, De Martini and Pearson (1982) was used, with some modification. Venous blood was collected into EDTA (final concentration 2.7 mg/ml). Following centrifugation at 3000 rpm (1800 G) for 20 minutes at 12°C, the buffy coat was collected into 30 ml phosphate buffered saline (PBS) and layered onto 3 ml Ficoll-Isopaque (specific gravity 1.077), in 15 ml tubes. These were centrifuged for 30 minutes at 2000 rpm (800 G) at room temperature, and the cells at the Ficoll-Isopaque/saline interface collected and centrifuged; where necessary, contaminating erythrocytes were lysed by resuspending the cell pellet in a solution of 0.8 per cent NH_4Cl, 0.1 per cent Na_3-EDTA, 0.01 per cent KH_2PO_4, pH 7.0. The lymphocytes were washed three times in PBS, at 600-900 G to remove platelets, and a sample was suspended in 0.05 per cent Trypan Blue and counted in a haemocytometer. The cells

were resuspended at a concentration of 2.0×10^6 viable cells/ml in 'Cell Culture medium' (10% foetal calf serum (CSL., Australia) in Dulbecco's modified Eagles medium (General Biochemicals, U.S.A.), to which was added 10^{-4} M 2-Mercapto-ethanol, 100 U/ml Penicillin, 100 µg/ml Streptomycin, 100 µg/ml Neomycin and 10 mM HEPES buffer (Sigma, U.S.A.)).

G. EMBRYONIC CULTURE TECHNIQUES

Twenty-seven embryos were removed from ewes between 18 and 29 days of gestation. Each was immersed in one of four media and placed in one of two culture systems. Aseptic conditions were used throughout the study.

The media used were:

(i) sheep serum: blood was centrifuged immediately after collection and the fibrin clot allowed to form in the separated plasma. Serum obtained by this method has been shown to result in better growth of early rat embryos than does serum obtained after clotting of the whole blood (Steele, 1972; Steele and New, 1974). The serum was heat-inactivated at $56^{\circ}C$ for 30 minutes as this has been reported to improve its capacity to support embryonic growth (Steele and New, 1974), and stored at $-20^{\circ}C$.

(ii) fifty per cent sheep serum in either Medium 199 (CSL, Melbourne) or Dulbecco's modified Eagles medium (General Biochemicals, U.S.A.).

(iii) Hanks' blanced salt solution (Hanks' BSS).

Some characteristics of these media are compared with those of foetal fluids in Table 1.1. Measurements of in vitro

pH were made on a Corning pH meter 125 (Corning, Stone, England).

The culture systems used were:

(i) a continuous flow system, in which a 60 ml plastic syringe with eccentric Luer tip (560S-E, Monoject, St. Louis, U.S.A.) served as the culture chamber and was connected by silicone rubber tubing of external diameter 4 mm (LKB-Producter AB, Sweden) to a 20 ml glass reservoir and to a 1200 varioperpex peristaltic pump (LKB-Bromma, Sweden). Gas was continuously supplied to the bottom of the reservoir chamber, and bubbled through the medium which was pumped through the culture chamber.

(ii) a roller system, in which 20 ml Universal bottles containing ten ml of medium were screwed into a rotating plastic drum, powered by a Parvalux electric motor (Bournemouth, England) and rotating at 47 rpm. Gas was continuously supplied to the drum, and thus to the medium.

In all cases the gas was five per cent CO_2 in oxygen (Carbogen, CIG, Australia), supplied to the medium at a rate of 100-200 ml/minute after being warmed and humidified by bubbling through water at 37°C. Since normal body temperature of the adult sheep is 39.1 $\pm$ 0.8°C (Clawsol, 1928), cultures were set up at either 36-37°C or 37.5-41°C.

Embryos were examined every three to six hours under a dissecting microscope for viability - the criterion for which was a microscopically visible heart beat. The medium was renewed at each examination.

H. TISSUE CULTURE TECHNIQUES

1. Vero cell culture: African Green Monkey kidney (Vero) cell monolayers (kindly supplied by Department of Microbiology, A.N.U.) were used for growing virus and for controls for this virus preparation; for biological assays for virus presence, virus titre, and specific neutralizing antibody; and for virus-positive controls for fluorescent staining of antigen and electron microscopy. The cell line was maintained in burlers, in growth medium consisting of Medium 199 containing 2.5 per cent bovine serum and 2.5 per cent foetal calf serum (pH 7.4), and passaged weekly as follows:

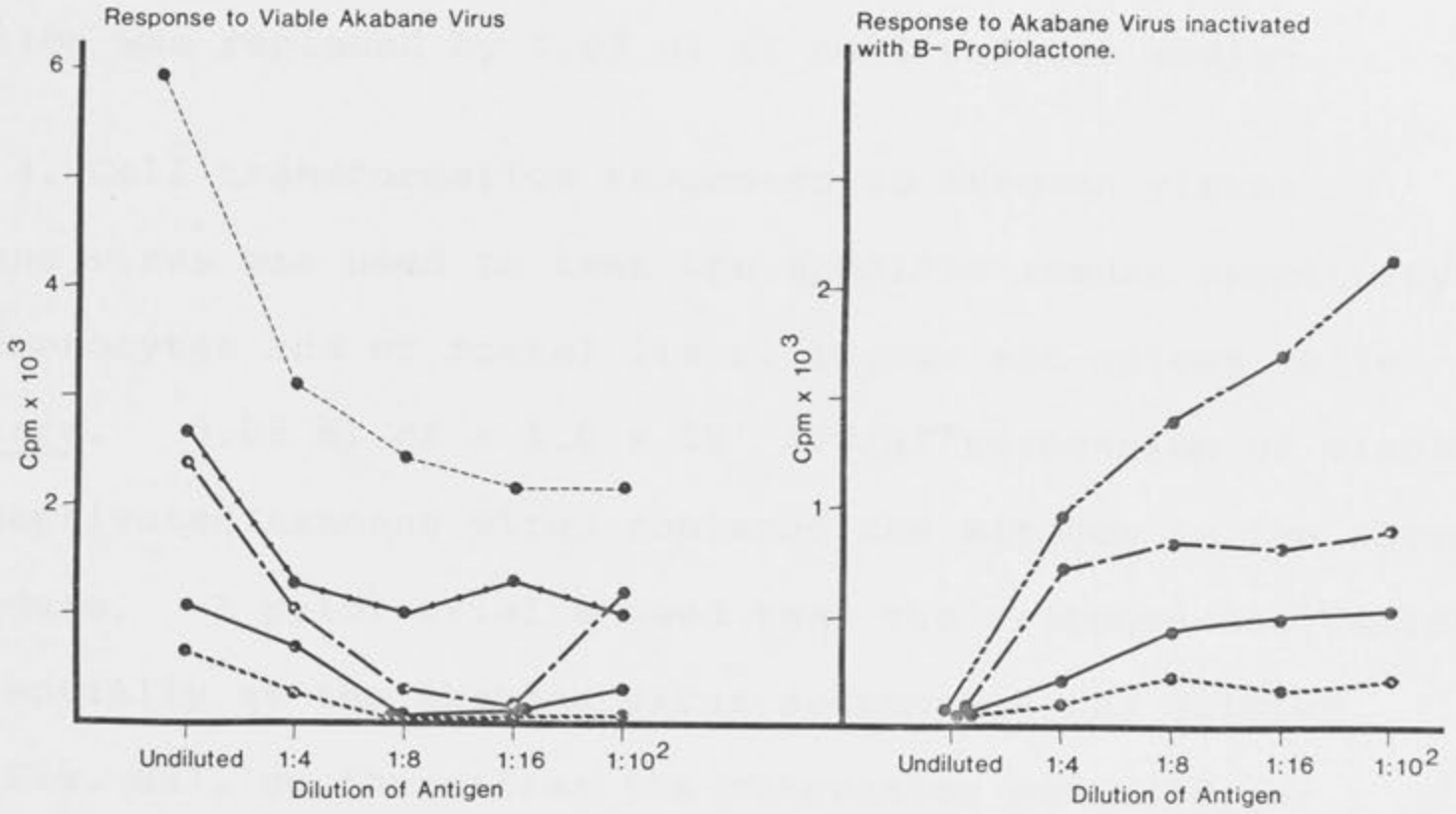
The medium was decanted and replaced with 24 ml of a Trypsin-versene solution*. After five to ten minutes at 37°C the cell sheet was dislodged, the fluid containing the cells was recovered and centrifuged for two minutes at 1000 rpm (200 G). The supernatant was discarded, and the cells re-suspended in growth medium and counted using a haemocytometer. Burlers were seeded with 1.0×10^7 cells in 250 ml medium. 50 mm glass or plastic (Kayline Plastics, South Australia), tissue culture petri dishes were seeded with 1.7×10^6 cells in five ml of medium, while 90 mm dishes were seeded with 5.0×10^6 cells in 15 ml medium: monolayers were confluent 24 hours later. Where Vero cells were confluent and needed to be maintained only, maintenance medium consisted of Medium 199 with one per cent foetal calf serum and 15 mM HEPES (pH 7.4).

* 0.025% Trypsin, 0.02% Versene, 0.025% $NaHCO_3$, 0.8% NaCl, 0.04% KCl, 0.006% Na_2HPO_4, 0.006% KH_2PO_4.

2. Mixed lymphocyte culture (MLC): This method is a variation of that of Urbianak, White, Barclay, Wood and Kay (1978), the changes being the use of slightly higher cell numbers and a different culture medium. Foetal liver and thymus were collected aseptically, passed through fine sieves, and after 24 hr culture in cell culture medium (as above), were resuspended in fresh medium at a concentration of 2.0×10^6 cells/ml. No attempt was made to purify the foetal cells, and the proportion of the cells assayed which was lymphoidal is therefore unknown. Adult peripheral blood lymphocytes were prepared as described and suspended in cell culture medium at a concentration of 2.0×10^6 cells/ml. In the MLC, the foetal cells were the responders, and the adult cells served as stimulators. In the reverse MLC, in which foetal cells were assayed for their capacity to stimulate rather than to respond, the foetal cells were the stimulators, and were irradiated to prevent their proliferation. Cells to be used as stimulators were exposed to 1500 rad gamma irradiation from a ^{60}Co source. 0.1 ml of each of the stimulator and responder cell suspensions were mixed in a Nunc (Denmark) microplate with flat-bottomed wells. Two controls were incorporated: responder cells with no stimulators, and responder cells cultured with stimulator cells from the same individual.

3. Cell transformation responses to mitogens: The technique used was that of Fiscus et al (1982) with some modifications. Concanavalin A (Con A) (Sigma), lipopolysaccharide B (LPS) from E. coli 0128:B12 (Difco), and phytohaemagglutinin (PHA), from Phaseolus vulgaris Type 5 (Sigma), were used as mitogens to test the non-specific immune reactivity of lympho-

Fig 0.3. Effect of dilution of antigen on the in vitro response to that antigen of adult peripheral blood lymphocytes from control and infected ewes.



—--14 ——99
-----27 -·-·-36
·—·—31 ----- 26

Sheep #14 and #99 were not inoculated. The remainder were inoculated I/V with viable Akabane virus, and cells collected for assay at nine days after inoculation.

cytes and foetal liver, spleen and thymus cells in vitro. Cultures were initiated by the addition of 2.0×10^5 cells (shown in pilot trials to be optimal) in 0.15 ml of cell culture medium (as above) to 0.05 ml of one of the mitogen solutions. The mitogens were used at final concentrations shown to be optimal in pilot trials, i.e. five µg/ml Con A, 50 µg/ml LPS, 50 µg/ml PHA. In control wells the mitogen solution was replaced by 0.05 ml of cell culture medium.

4. Cell transformation responses to Akabane virus: Akabane virus was used to test the specific immune reactivity of lymphocytes and of foetal liver, thymus and spleen cells in vitro. 0.05 ml of a 1.0×10^7 PFU/ml suspension of viable or inactivated Akabane virus replaced the mitogen in the above procedure. A pilot trial showed that the response decreased exponentially as the Akabane virus suspension was diluted (see Fig. 03), so thereafter the suspension was used undiluted. In case of the wells to which both Con A and virus were to be added, cells were suspended in 0.1 ml of medium, so that the total volume should remain at 0.2 ml. Two controls were incorporated: in one the virus suspension was replaced by 0.05 ml of cell culture medium, in the other it was replaced by 0.05 ml of the Vero control suspension.

5. Determination of cell proliferation in mixed lymphocyte culture and transformation assays: The method used was that of Urbianak et al (1978). Cultures were incubated in a humidified atmosphere of ten per cent CO_2, seven per cent O_2, and 83 per cent N_2. After two or four days in culture, the cells were labelled with one µCi/well, of methyl ^{3}H-thymidine

(specific activity 5.0 Ci/mmol, New England Nuclear), and then cultured for a further 18 h before being harvested on to glass fibre filters with a Titertek automatic sample harvester (Flow Laboratories). Each filter disc was placed in a vial with five ml of 0.5 per cent 2,5-Diphenyloxazole (PPO, Koch-Light Labs., Colnbrook, England) in xylene, and the sample was then counted in a Packard Tri Carb liquid scintillation spectrophotometer for one minute. All cultures were performed in triplicate, and the results given are the means expressed as counts per minute (Cpm) and/or as standard indices (SI). In the case of MLC,

$$SI = \frac{\text{Cpm of stimulated cultures}}{\text{Cpm of self-stimulated cultures.}}$$

In the case of mitogen stimulated transformation assays,

$$SI = \frac{\text{Cpm of stimulated cultures}}{\text{Cpm of non-stimulated cultures.}}$$

In the case of virus stimulated transformation assays,

$$SI = \frac{\text{Cpm of virus-stimulated cultures}}{\text{Cpm of Vero cell-stimulated cultures.}}$$

Standard errors were calculated but, to avoid confusion, are not included in tables of results. Maximum standard errors are given with the first table in each experiment.

6. Culture of foetal organ fragments: Liver, spleen and thymus were taken from control foetuses and from foetuses inoculated with allogeneic cells (adult efferent lymphocytes) between days 31 and 59. Fragments of these organs, approximately one mm^3 in size, were placed in six-well plastic culture dishes (Lindbro, Flow Laboratories) in medium consisting of 20 per cent foetal calf serum in Dulbecco's modified Eagles medium, 100 U/ml Penicillin, 100 µg/ml Streptomycin

and 100 µg/ml Neomycin, and cultured for seven days in a humidified Carbogen atmosphere (10% CO_2 in O_2). Special gas (10% CO_2, 7% O_2, 83% N_2) as used for lymphocyte cultures was tried, but was less successful. Tissues were then fixed in formal saline and prepared as above. They were examined histologically for cell survival and/or proliferation, and maintenance of tissue architecture.

7. In vitro Akabane infection of foetal efferent lymphocytes: Efferent lymphocytes from two uninfected foetuses aged 133 and 136 days were cultured in cell culture medium (1.0×10^6 cells in 2 ml medium/well) in six-well tissue culture trays (Lindbro), in (a) the absence and (b) the presence of 8.0×10^5 PFU of Akabane virus/well, for two, four or six days. Cells were then harvested after which they were examined by electron microscopy for the presence of intracellular virions, and by plaque assay for the virus titre. In the latter case, all samples, including the original inoculum, were exposed to ultrasound from a Sonifer B-12 for five seconds, frozen in liquid nitrogen and stored in liquid nitrogen vapour until all were titrated at the same time by plaque assay. This was because the titre of the virus with which the cultures were infected was halved by freezing, therefore all samples were frozen once to allow comparison.

I. HISTOLOGICAL PROCEDURES

1. Preparation of cryosections: Small pieces (4 mm^3) of foetal tissues taken at post-mortem were placed in plastic moulds (Cryomold, Miles Laboratories, Naperville) with Tissue-Tek O.C.T. Compound embedding medium (Miles Laboratories),

snap frozen in a liquid nitrogen-cooled heat sink, and placed in liquid nitrogen until stored in a liquid nitrogen vapour freezer. 8 µm sections of these blocks were cut on an Ames Cryostat II (Miles Laboratories) at -20°C.

2. Preparation of paraffin sections: Foetal tissues taken at post-mortem were fixed in formol saline for a minimum of two days (10% formaldehyde, 0.9% saline, 0.04% NaH_2PO_4 H_2O, 0.65% Na_2HPO_4, in distilled water). Tissues were then dehydrated by successive immersion for one hour in 70 per cent alcohol, 80 per cent alcohol, 90 per cent alcohol, 100 per cent alcohol (6 changes), for two hours in chloroform (2 changes) and paraffin (2 changes). Tissues were then embedded in paraffin (melting point approximately 52°C). When set, 4 µm sections were cut on a Sorvall JB-4 Microtome (Du Pont, Connecticut, U.S.A.) and stained with haematoxylin-eosin.

3. Preparation of uterine sections: After ventral mid-line laparotomy of the ewe, the uterus was exposed, and 0.9 per cent saline containing 40 U/ml heparin was infused into both middle uterine arteries. This was followed by fixative (4% formalin in 0.2 M phosphate buffer, pH 7.4) and then by a suspension of carbon in a cold fluid gelatin mass. This mass was prepared by the method described by Guyer (1953). Five g of gelatin was dissolved in 100 ml of tepid distilled water, the solution was coloured to the desired shade with India ink and five g of potassium iodide was added slowly. The mass remains fluid at ordinary temperatures, but when injected objects are placed in five per cent formalin it sets and is thereafter unaffected by reagents. The uterus and ovaries

were then removed intact, fixed in five per cent neutral buffered formalin for 24 hours, then ten per cent formalin for four days. The uterus was then filled with three per cent agar to minimize disturbance of foetal membranes during further processing, embedded in paraffin as a single large block, and sectioned with an LKB Multirange Microtome. Longitudinal sections, five µm thick, were cut through the pregnant horn until the embryo was reached. The sections were stained with haematoxylin-eosin and examined under the light microscope for placental histology; the foetal and maternal blood vessels were differentiated by the presence of carbon in maternal vessels only.

4. Preparation of sections for electron microscopy: Ten ml samples of lymph from the cannulated prescapular efferent lymphatics were collected twice daily, centrifuged at 600 G for ten minutes, washed and fixed in three per cent gluteraldehyde in 0.1 per cent cacodylate buffer, pH 7.4, for three hours. The cells were then post-fixed in one per cent osmium tetroxide in 0.1 per cent cacodylate buffer, pH 7.4, for 1.5 h , and en bloc stained in one per cent aqueous uranyl acetate for 1.5 h .

Samples of the viral preparation injected into the foetuses were prepared for electron microscopical examination as a viral morphology control. They were concentrated by filtration in a Minicon concentrator B125 (Amicon, Danvers, U.S.A.) and spun through three per cent molten agar until set. Cell pellets and agar blocks were cut into 0.5 mm^3 blocks, dehydrated, and placed in 50/50 absolute ethanol/Spurr epoxy-

resin (Spurr, 1969) for three hours, then 100 per cent Spurr for a further three hours, and finally embedded. Sections were taken from three levels of each block, cut on an LKB Ultratome III and stained with 'Reynolds' lead citrate. A total of 200 cells per block were examined, and some of these photographed, on a Philips 301 Electron Microscope, for the presence and number of intracellular virions, and for cellular pathology.

Normal foetal efferent lymphocytes and efferent lymphocytes from an Akabane-infected foetus were cultured in vitro in the presence of Akabane virus and processed similarly.

Four twenty-two day old embryos were prepared for electron microscopy after they were each cut into approximately 12 cubes to permit adequate fixation. Thereafter preparation of the sections was as described for the lymphocytes. Blocks containing nervous tissue and undifferentiated mesenchyme were selected from thick sections, and from five sections of each embryo a minimum of 200 cells of each of these tissue types was examined by electron microscopy for the presence of Akabane virions.

5. Preparation of blood smears: Thin smears were prepared from whole blood collected by jugular venous cannula from two foetuses infected with Akabane virus (#75 and #63). These smears were stained with "Diff-Quik" modified Wright's stain and examined by light microscopy for differential leukocyte counts.

J. AUTORADIOGRAPHY

1. Labelling of allogeneic efferent lymphocytes: Allogeneic adult efferent lymphocytes were collected from ewes which had undergone popliteal efferent lymphatic duct cannulation as described in B.4. They were labelled by a method modified from that described by Ford (1978). The cells to be labelled were incubated with 20 µCi methyl-^{3}H-thymidine/ml cell suspension for one hour at 37^{o}C in a humidified CO_2 atmosphere. They were then washed three times in Hanks' BSS, and resuspended in Hanks' BSS to a concentration of 1.0×10^{9} cells/ml before being injected into the foetal umbilical vein (3.0×10^{8} cells/foetus).

2. Preparation of autoradiographs: This method was selected from those described by Stein and Yanishevsky (1979). The first group of foetuses involved were those from Akabane infected ewes, described in Chapter 5. Fifty µCi methyl-^{3}H-thymidine was injected into the umbilical vein of each foetus two hours before its recovery. Gelatin-coated (0.5% gelatin with 0.05% chrome alum, in distilled water, to a thickness of 1.0×10^{-3} - 1.0×10^{-2} µm) paraffin sections of tissues taken at post-mortem were dewaxed by immersion for ten seconds in each of the following in turn: xylene, absolute alcohol, 90 per cent alcohol and 70 per cent alcohol, and rinsed for 30 minutes in tapwater. In a darkroom illuminated by a safelamp fitted with a No. 1 (red) Kodak Safelight Filter and a 25 W pearl lamp, a stripped emulsion layer (Kodak Fine Grain Autoradiographic Stripping Plate AR.10) was floated onto each section, dried and placed in a light-proof box for exposure for six to ten weeks at 4^{o}C. Specimens were developed in

Kodak D-19 Developer for 4.5 minutes at 20°C, rinsed for one minute in water at 20°C, then fixed for seven minutes at 20°C in Hypam Rapid Fixer (Ilford). After washing for 15 minutes in running water, the specimens were stained with Azure-A (0.1% in acetate buffer, pH 5.2) for ten seconds, rinsed three times in tap water and dried. During developing and staining, care was taken that the emulsion was not disturbed. When examined by light microscope, the black grains then overlaid the labelled thymidine incorporated into cellular DNA.

Autoradiographs were also prepared from foetuses described in Chapter 2. Foetuses inoculated with radioactive-labelled allogeneic cells were fixed in formol saline and embedded in paraffin. Autoradiographs of the sections were prepared as above and examined microscopically for location of injected cells.

K. STATISTICS

The significance of difference in means of various populations was assessed using Student's t-test.

The error bars shown on Figures 0.2 and 3.3 represent standard errors.

Results are presented in tables, figures and plates and summarized in the text. In the tables, a blank indicates that the test was not done, and a dash that the results were negative, while positive results are indicated by pluses or numerals as indicated in footnotes.

CHAPTER 1

ACCESSIBILITY OF THE FOETAL LAMB TO AKABANE VIRUS

CHAPTER 1 - ACCESSIBILITY OF THE FOETAL LAMB TO AKABANE VIRUS

1.1 INTRODUCTION

The overall objective of this project was to examine the relevance of immunological maturation of the foetal lamb for its responsiveness to Akabane virus. In achieving this, it seemed likely that, apart from direct measurement of foetal performance in the various modalities of the immune response, it should be possible to draw some inferences about the development of its immunological capabilities by observation of any pathological changes resulting from viral exposure. As these changes would effectively represent the outcome of interaction between viral pathogenic processes and host response, their description should indirectly assist in understanding the latter.

One qualification that appeared likely to apply to interpretations of foetal responses after maternal infection concerned possible variations in accessibility of the embryo or foetus at different stages of its development to the virus. Both the occurrence and the extent of foetal exposure to a maternally administered virus would be likely to be influenced by the immune status of the mother and the morphology of the placental structures separating the ewe from the lamb. The first obstacle to interpretation of the results could be variation introduced because of immune elimination or inactivation of virus before an opportunity arose for it to enter the foetus. This risk could be eliminated by restricting experimentation to seronegative ewes. To eliminate

variations introduced in the process of transmission of virus from mother to foetus was much more difficult. This consideration influenced the design of some of the subsequent experiments, as for example those in which lambs infected via the ewe were compared with directly inoculated subjects. Two types of experiment were undertaken at an early stage of the project, to test possible ways in which variability in virus transmission, both between ewes and at different stages of gestation, could be eliminated or accounted for. These experiments involved, respectively, the cultivation of the ovine embryo *in vitro* and the delineation of the process of apposition of maternal and foetal tissues.

Trial of sheep embryo culture

1.1.A INTRODUCTION

Little information is available about the capacity of the ovine embryo to respond to foreign substances during its first month after conception. Nevertheless, as differentiation of the major organ systems is substantially completed by 21 days (Green and Winters, 1945) it is likely that some development of a rudimentary immune system has commenced in the pre-implantation lamb. The possibility that sheep embryos could be transferred to, and maintained in, tissue culture was appealing as this technique could obviate the very substantial difficulties encountered in gaining repeated access to such embryos *in vivo* for purposes of antigenic challenge, observation and sampling of tissues. Although fertilized sheep ova have been successfully cultured up to the early blastocyst stage (Tervit, Whittingham and Rowson, 1972), no attempts to culture older embryos have been reported. There have been

reports of successful *in vitro* culture of other mammalian embryos during organogenesis. Rat embryos of eight to 14 days gestation, that is after implantation, have been grown for up to 4.5 days (New, 1978; New and Coppola, 1977). Post-implantation, early somite stage mouse embryos have been successfully cultivated for two days (Clarkson, Doering and Runner, 1969) and opossum embryos explanted between 7.5 days and birth (12.75 days) have been maintained for 24 hours (New, Mizzell and Cockroft, 1977). The most notable difference between these species and the sheep at this age is that implantation is relatively delayed in the sheep. Consequently, unlike most other species, major organogenesis has been completed in lambs before implantation. While there was no indication whether the sheep embryo would develop *in vitro* as readily as did the rat, the potential usefulness of this approach for the present project justified a trial. Accordingly, a pilot trial was established to determine whether the embryonic lamb of 18 to 30 days gestation could be cultivated *in vitro* for a sufficient length of time to permit experimentation. Five days was judged to be the shortest period in which any response by the embryo to antigenic challenge was likely to be detectable.

1.2.A EXPERIMENTAL DESIGN

Twenty-seven embryos were recovered from normal ewes as described in Materials and Methods, and placed in culture. They ranged in age from 18 to 30 days. Each was placed in either a culture system in which the bottle containing the embryo and medium was rotated, or one in which the container remained stationary while the medium was continually pumped

through it (see Materials and Methods). This allowed comparison of two methods of maintaining the oxygen content of the media in contact with the embryo. Each embryo was allotted one of four media - sheep serum, Hanks' balanced salt solution, 50 per cent serum in Medium 199, and 50 per cent serum in Dulbecco's modified Eagles medium. The aim was to establish whether any type of medium was superior to the others in its ability to maintain embryonic viability. Each culture was maintained at a temperature between 36°C and 41°C inclusive, a range based on the normal rectal temperature of the adult ewe (39.1°C, Clawsol, 1928). Six animals were placed in the continuous flow system. Once it became apparent that survival time of embryos in the two culture systems was similar, the remaining embryos were cultivated in the roller system as access to, and therefore observation of, the embryo was more satisfactory. Similarly, after all of the six embryos aged 18 to 21 days, which were cultured at temperatures higher than 37°C died within three to ten hours of explantation, remaining embryos were maintained at 36 to 37°C. The distribution of embryos among the other culture conditions is summarized in Table 1.2. Embryos were examined every three to six hours, and when cessation of heartbeat was observed, the survival time was estimated to be the midpoint between that time and the time of the preceding observation at which a heartbeat was present.

1.3.A RESULTS

Table 1.1 compares the measured osmolarity and pH of the media used in this experiment before and after culture of the embryos, with reported values for foetal fluids of embryonic

TABLE 1.1 - Comparison of some characteristics of foetal fluids and culture media.

Sample	Osmotic pressure (mosmol/h)	pH pre-culture	pH post-culture for 3h
Hanks BSS	288 ± 2	7.4	6.4
[a]100% serum	319 ± 1	8.9	8.1
[a] 50% serum in medium	297 ± 1	8.3	7.6
[b]allantoic fluid at 22d	231 ± 7	7.5	
[b]allantoic fluid at 31d	214 ± 8	7.4	
[b]amniotic fluid at 31d	293 ± 3	7.4	

a = sheep serum, in vitro measurements.

b = in vivo values for sheep reported by Wales and Murdoch, 1972.

sheep of a similar age. The mean survival times of embryos subjected to different culture conditions is summarized in Table 1.2. As there was no difference in survival of animals in the continuous versus the roller system, results from these two groups have been amalgamated.

The age of the embryos appeared to influence their capacity for survival, with younger embryos surviving longer in culture. This was in agreement with similar observations of rat embryos (New, 1978). The results of the group of embryos explanted between 18 and 24 days were then examined separately so that temperature and media differences could be compared. Survival times were greater in animals maintained at 37^{o}C than in those at higher temperatures. The medium associated with the longest mean survival time was Hanks' balanced salt solution.

Before explantation, the average pulse rate was 80 beats/minute. During culture, rates varied between 20 and 40 beats/minute. After its cessation, the heartbeat of several of these embryos was restarted using cardiac massage. It then continued to beat regularly at a normal rate for up to 19 hours. This resuscitation was effective on only one occasion for each embryo. Heartbeat had ceased by 48 hours after explantation in all cases.

1.4.A DISCUSSION

The intervals between observations were such that survival times could not be estimated with an accuracy greater than three hours. However, as the aim of the trial had been to test the feasibility of cultivation for periods of the order

TABLE 1.2 - Embryo survival under various culture conditions.

Conditions		Mean survival time (h)	n	
Age:	18-24d	15.4	16	11.9
	25-30d	5.2	11	1.5
18-24d				
Medium:	Hanks BSS	25.9	4	12.2
	100% serum	11.1	6	9.0
	[a]50% serum in medium	14.8	6	10.2
	[b]50% serum in M199	10.4	4	9.6
	[b]50% serum in DMEM	23.5	2	3.0
Temperature:	36-37°C	18.2	11	11.7
	37.5-41°C	9.4	5	8.9

M199 = Medium 199

DMEM = Dulbecco's modified Eagles medium

serum = sheep serum

a = this group is the combination of groups b.

of five days, this was not a significant limitation. If this preliminary study were to be extended, a large number of animals would be required especially if a multifactorial experimental design were adopted. The first reason for this requirement would be the wide variety of cultural conditions to be examined; the second is that apart from those variables which could be controlled in the laboratory, there are other variables to consider which it might not be possible to control fully. For example, embryonic genotypes would vary considerably in an outbred population such as the Merino while the effect of maternal factors such as nutrition, stress and disease, which influence early embryonic survival in vivo, may have been exerted before explantation and have continued to be operative in vitro.

The results suggest that in any continuation of this work, preferable conditions would include incubation temperatures not in excess of 37°C, media chemically buffered to a pH of 7.0 - 7.4, and explantation of embryos before 24 days of gestation. The study failed to establish superiority for any particular medium. The fact that survival was as good or better in a balanced salt solution as in complicated or serum-supplemented media suggests that it was more likely to be correlated with the endurance of the embryo rather than with any supportive attribute of the medium. Although it may have been possible to culture embryos younger than 15 days in which organogenesis had not occurred, such embryos are unlikely to have been of value for the purposes of this project.

Relationship of foetal and maternal tissue to each other at different stages of gestation

1.1.B INTRODUCTION

It has been speculated that failure to observe lesions in foetuses from ewes infected in the second half of pregnancy (Parsonson et al, 1977) may result from the development of a placental impediment to transmission of pathogenic virus to the foetal lamb. The onset of foetal susceptibility around 30 days may be related to some facilitation of viral transmission as a result of fusion of chorion and allantois at this time (Parsonson et al, 1981b). In view of these instances of demarcation of foetal susceptibility to Akabane virus at different stages of pregnancy, and the possibility that these changes could arise as a result of alterations in maternal/ foetal relationships at these stages, it appeared appropriate to examine some of the morphological aspects of implantation and placental development.

Fusion of foetal membranes with the maternal component of the placenta occurs at a substantially later stage of gestation in ruminants than in laboratory rodents or man. The ruminant placenta takes the form of multiple foci (placentomes) each of which consists of a maternally derived caruncle and a foetal cotyledon. Apposition of embryonic trophoblast and maternal epithelium is followed by loss or transformation of the caruncular maternal epithelium, which is replaced by a syncytial layer. There has been a long-standing controversy concerning the origin of this syncytial layer, with one group of authors claiming it to originate from the binuclear cells of the embryonic trophoblast (Davies and Wimsatt, 1966) and another considering it to be modified maternal epithelium (Steven, 1975). Some weeks later the intercaruncular epithelium is also transformed (Boshier, 1969). Interdigit-

ation of the maternal component of the placentome and the overlying foetal trophoblast then occurs, and when a sufficient degree of attachment is formed such that the blastocyst is not under normal circumstances displaced, implantation is said to have commenced (Steven and Morriss, 1975).

If experiments concerned with transplacental transmission of virus are to be interpreted in terms of the morphology of the tissues involved, the time of implantation is likely to be particularly important. Contact of the blastocyst with endometrial epithelium has been observed to occur between days ten and 17 (Green and Winters, 1945; Amoroso, 1952). Reports also vary in the times given for implantation of the ovine embryo from 22 to 31 days (Wimsatt, 1950; Assheton, 1906). To define this time more exactly, the histology of the normal embryonic-maternal junction at 24 to 29 days of gestation was examined by optical microscopy after preparation of the specimen in a manner designed to minimize disturbance of normal relationships.

1.2.B EXPERIMENTAL DESIGN

Three normal pregnant ewes were anaesthetized at 24, 26 and 29 days gestation, and their uterine blood vessels perfused with a carbon suspension to label maternal blood vessels. The uteri were then fixed and embedded whole and the pregnant horns sectioned and stained with haematoxylin-eosin for examination by light microscopy. As described in the Materials and Methods, the horn was filled with agar to prevent disturbance of the embryonic-maternal relationship. Additionally, samples of placentomes from 16 normal pregnant

ewes, ranging from 40 to 145 days gestation, were fixed, sectioned and stained for examination by light microscopy.

1.3.B RESULTS

Optical photomicrographs of the placentome and foetal membranes of the 29 day specimen are shown in Plates 1.1 and 1.2. The 24 day specimen had intact maternal endometrial epithelium in the intercaruncular regions, with loose apposition of foetal membrane. In the caruncular regions, no maternal or foetal villi were observed, and the caruncular surface was flat with intact non-columnar epithelium. The foetal trophoblast was loosely apposed to the epithelium, separated by an eosinophilic amorphous layer.

At 26 days, the intercaruncular regions were unchanged. The caruncular surface was still flat with no development of villi; in some areas the maternal epithelium was lost and the trophoblast appeared to be attached closely.

In the 29 day specimen (Plates 1.1 and 1.2) the maternal epithelium of the intercaruncular regions was intact but flattened. Areolae had begun to develop in the trophoblast overlying the endometrial glands. In the caruncular regions, maternal and foetal villi were observed, and the maternal epithelium was usually absent. Where epithelium was still present it had become flattened. The trophoblast appeared closely attached in some cases but the degree of adhesion varied between placentomes.

No structural differences were observed in the placentome specimens of 40-145 days gestation, although as the placenta

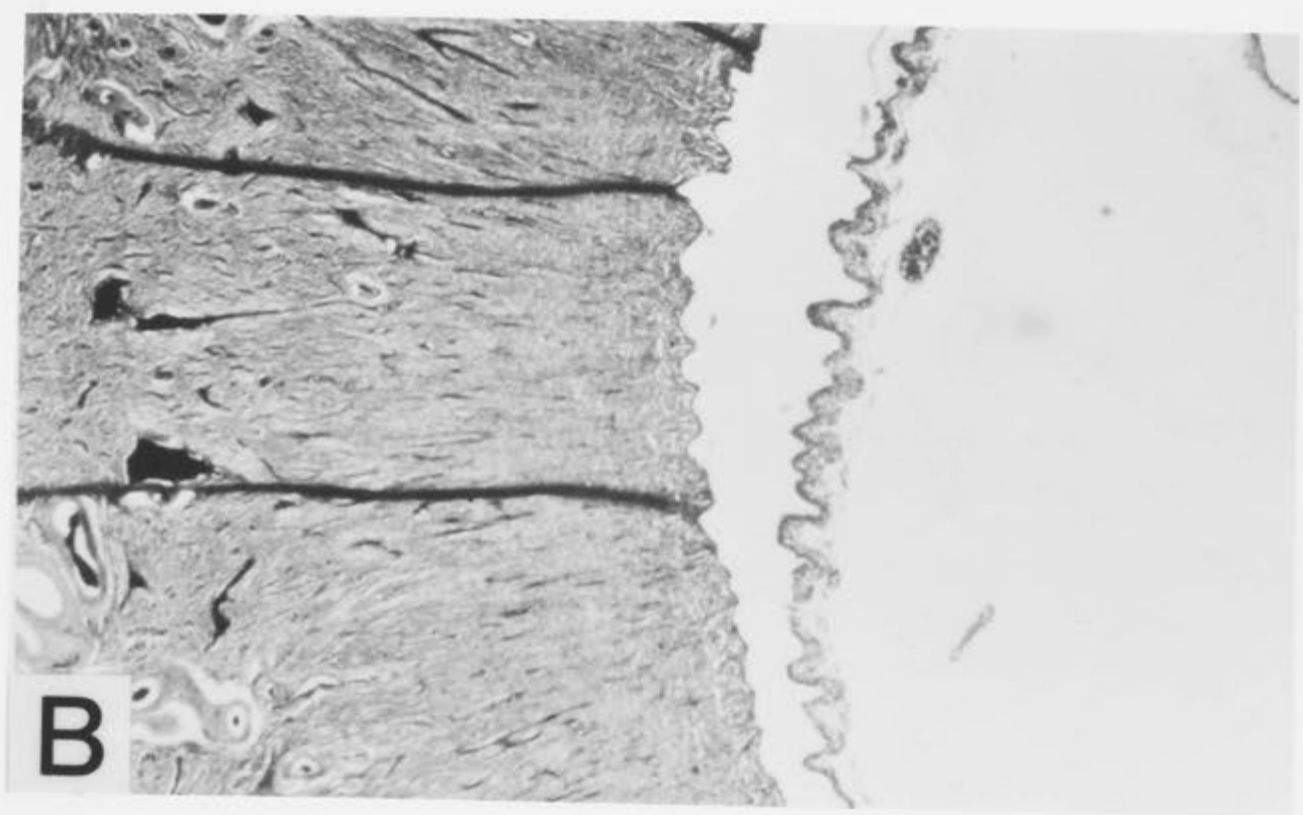
PLATE 1.1 Morphology of the ovine placenta at implantation (29 days gestation).

A: A complete section of the pregnant horn with the embryo visible in the centre. Maternal blood vessels, infused with carbon, appear black.
Haematoxylin-eosin, x 1.

B: The same specimen, showing a maternal caruncle and the overlying foetal trophoblast, with no interdigitation and loose apposition.
Haematoxylin-eosin, x 100.

C: Another maternal caruncle and overlying foetal trophoblast, showing interdigitation.
Haematoxylin-eosin, x 100.


A


B

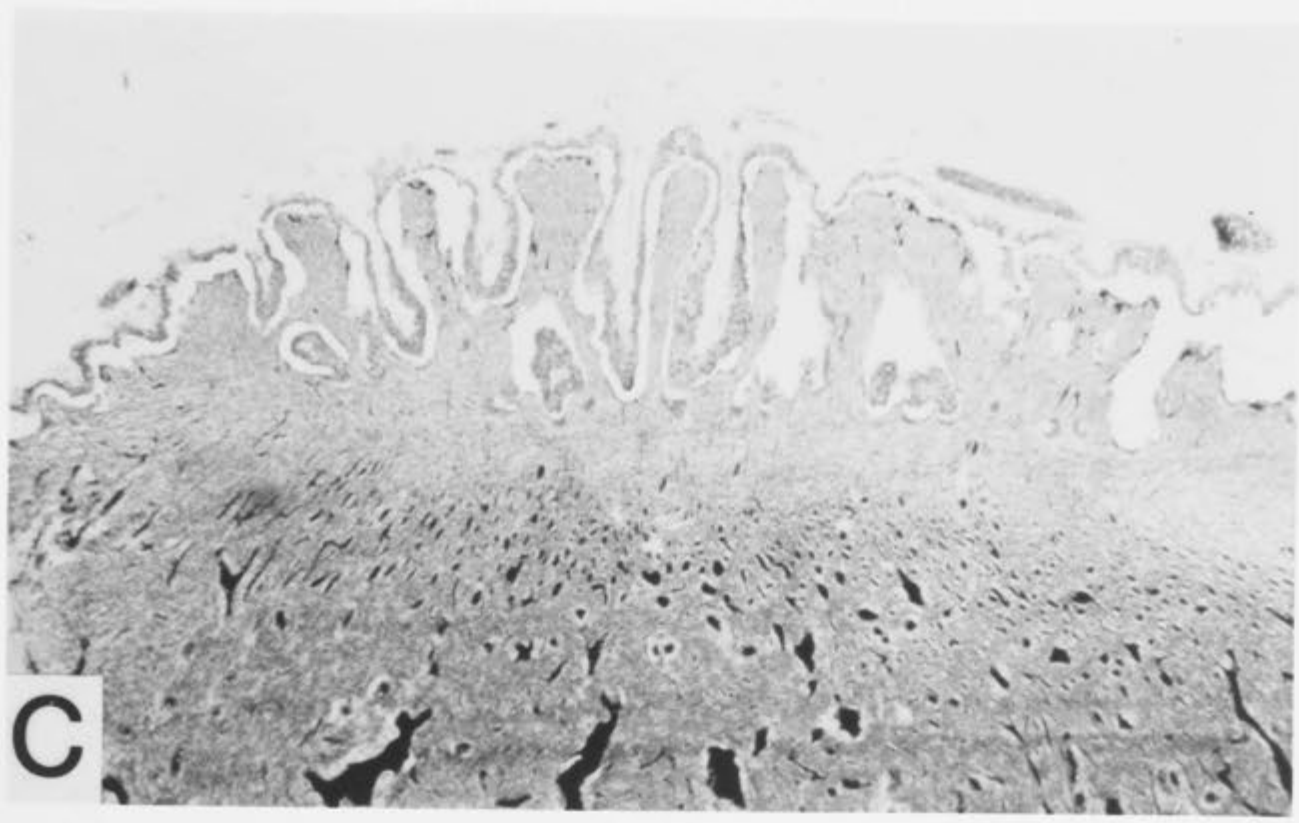

C

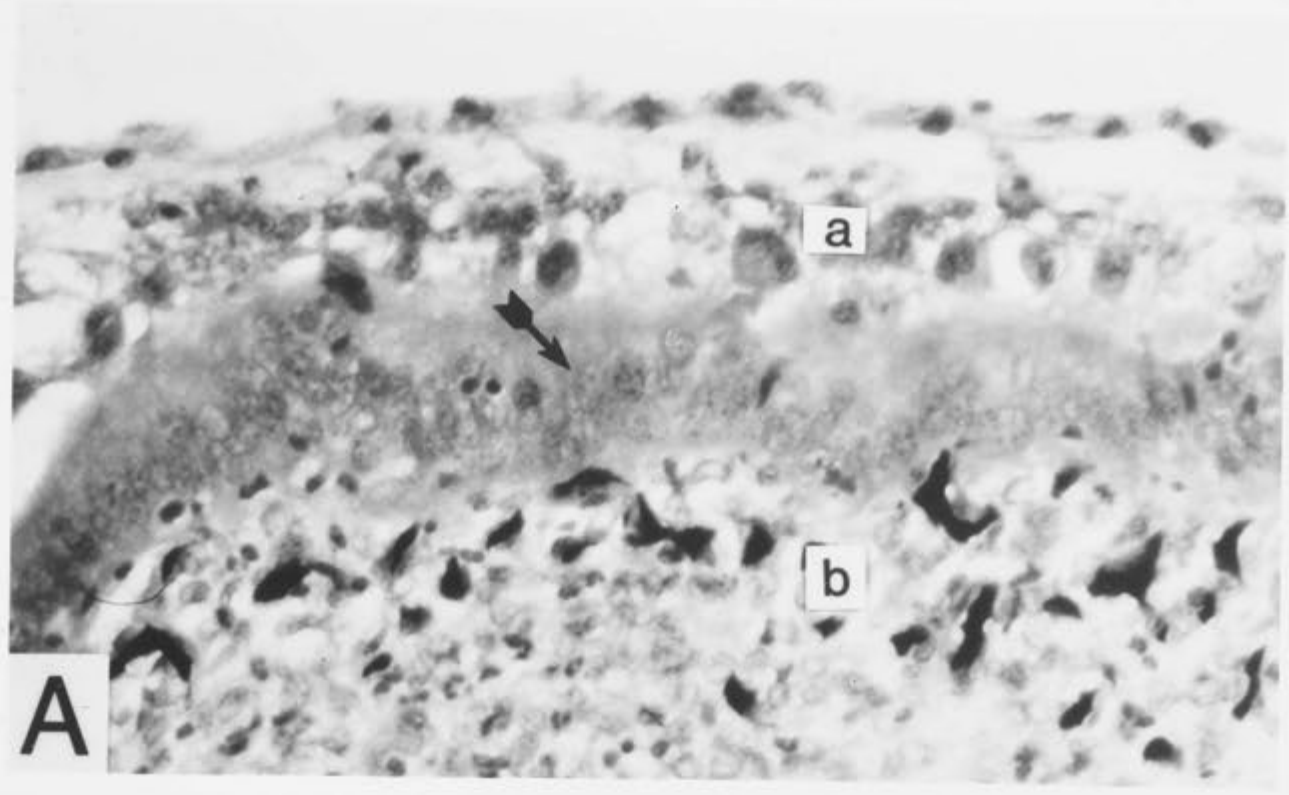
PLATE 1.2 Morphology of the ovine placenta at 29 days gestation.

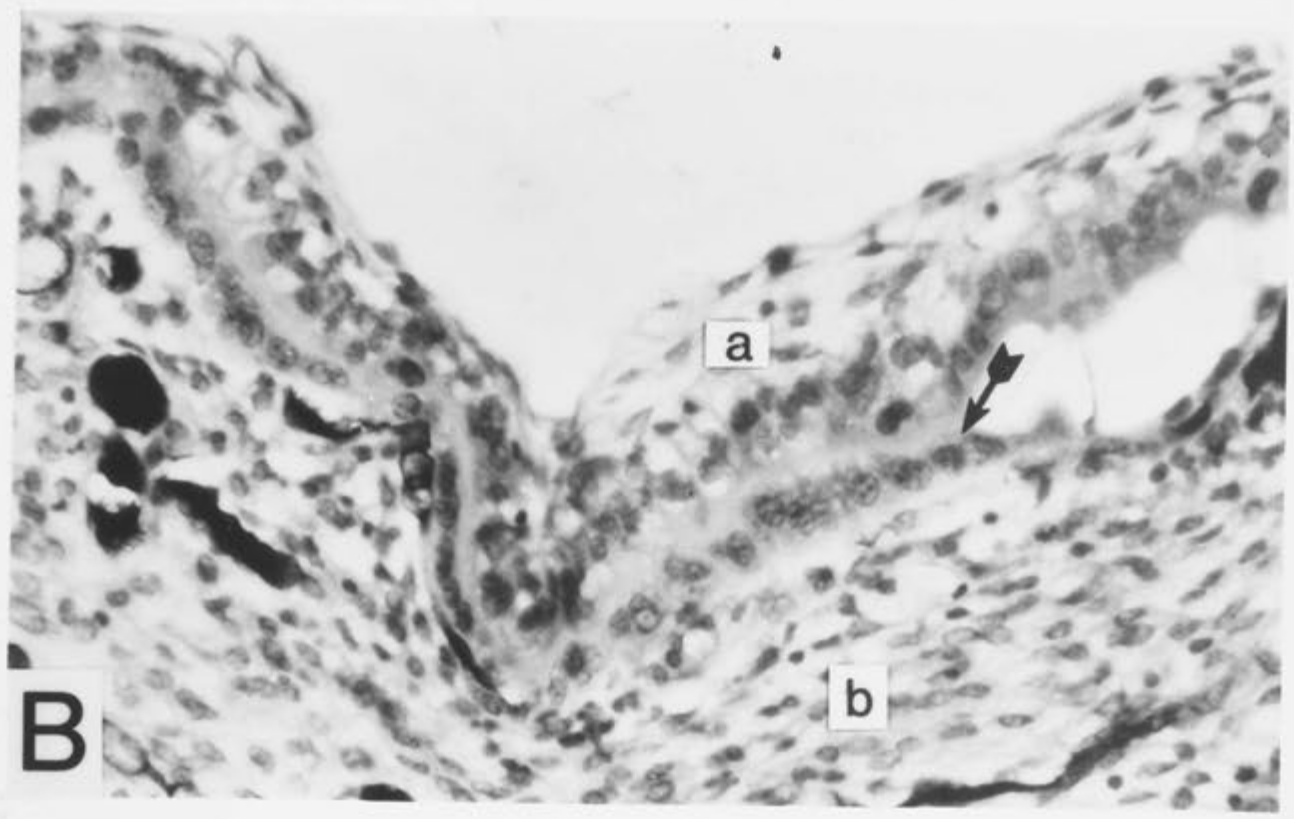
These are higher power views of the maternal and foetal components of placentomes from the specimen shown in Plate 1.1.

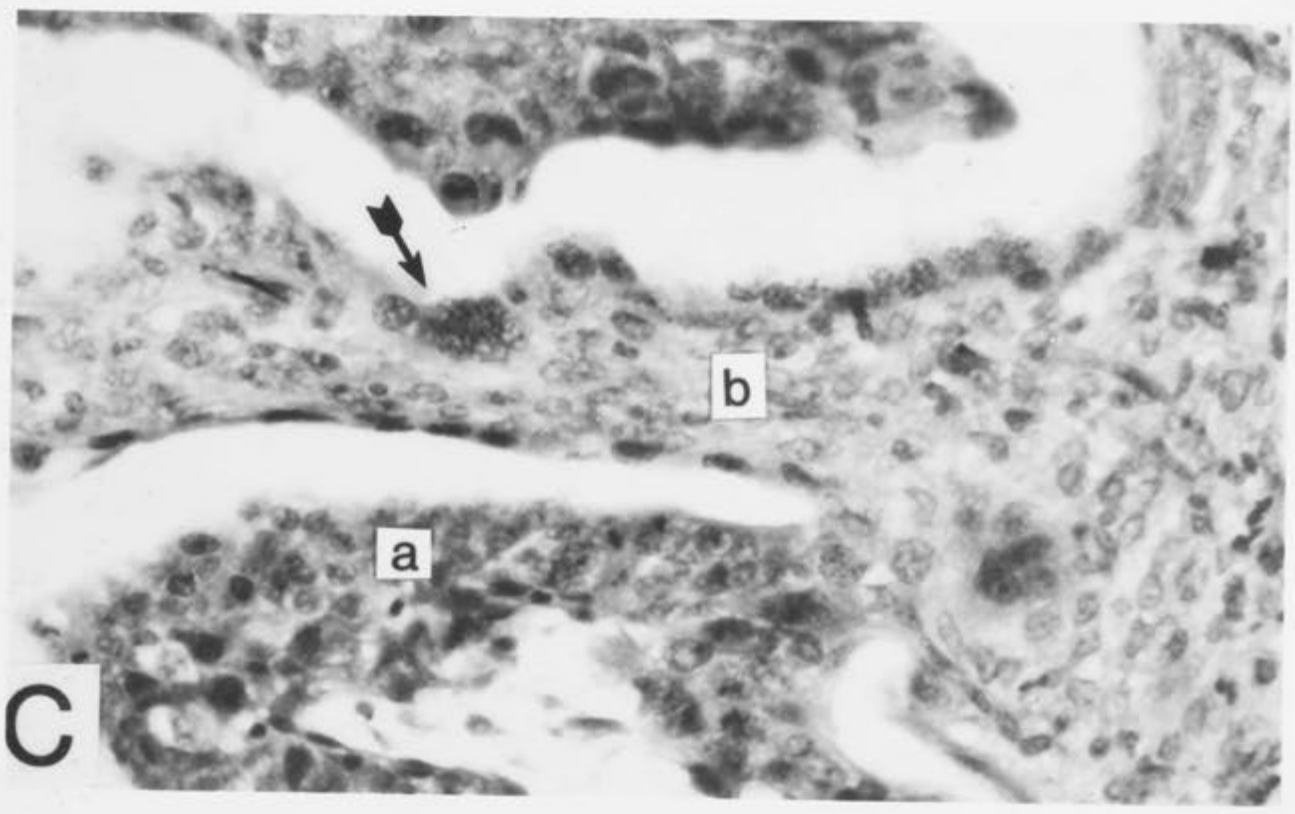
A: Part of a placentome. a = foetal trophoblast, b = maternal stroma. The maternal epithelium (arrowed) overlying the maternal caruncle is still columnar and intact but there is close apposition between it and the trophoblast. Carbon is visible in the maternal blood vessels. Haematoxylin and eosin, x 250.

B: An intercotyledonary region. a = foetal trophoblast, b = maternal connective tissue. The maternal epithelium is flattened but still intact and identifiable. An amorphous eosinophilic substance separated foetal and maternal cell layers. Haematoxylin and eosin, x 250.

C: Part of a different placentome. a = foetal trophoblast, b = maternal stroma. In some areas the maternal epithelium has been replaced by syncytial cells in monolayers or groups (arrowed). Some separation of trophoblast and syncytium has occurred in this section during processing. Haematoxylin and eosin, x 400.


a
b
A


a
b
B


b
a
C

matured the maternal inter-villous tissue was reduced. The 40 day placentome differed from the 29 day specimen chiefly in the degree of development of the maternal and foetal villi: villi were more numerous and more branched in the older placentome. There was also a difference in the syncytial layer lining the maternal crypt. At 29 days there were foci of nuclei replacing the maternal epithelium (Plate 1.2), but by 40 days these had given rise to a complete syncytial layer lining the maternal crypt.

Photomicrographs of placentomes of selected ages are shown in Plate 1.3. No morphological differences were observed between 40 and 145 day placentomes that could account for failure of virus transmission in late gestation. The foetal trophoblast became more flattened towards mid-gestation, and as the maternal villi became more branched the amount of tissue in each decreased, so that there was less tissue between maternal capillaries and foetal trophoblast as the placenta matured.

1.4.B DISCUSSION

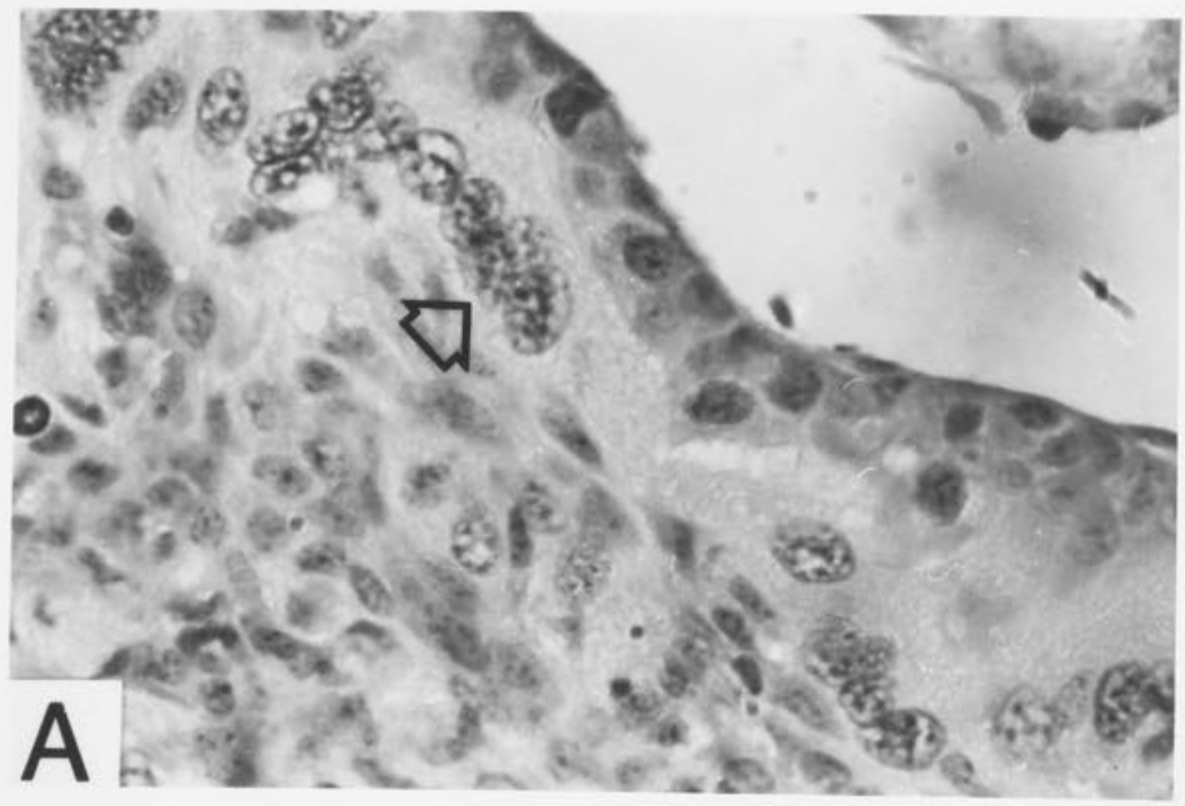
These results demonstrated that implantation commenced at approximately 26 - 29 days, but that it was not an all-or-none phenomenon. It appeared feasible that foetal-maternal contact from this time would suffice for the spread of a virus capable of replicating in both tissues, although definitive placental architecture was not attained until a time close to 40 days. Thereafter, no structural changes which appeared likely to preclude the transmission of the virus were observed. In fact, the thinning of the maternal inter-villous tissue

PLATE 1.3 Morphology of the normal ovine placentome over the first half of gestation.

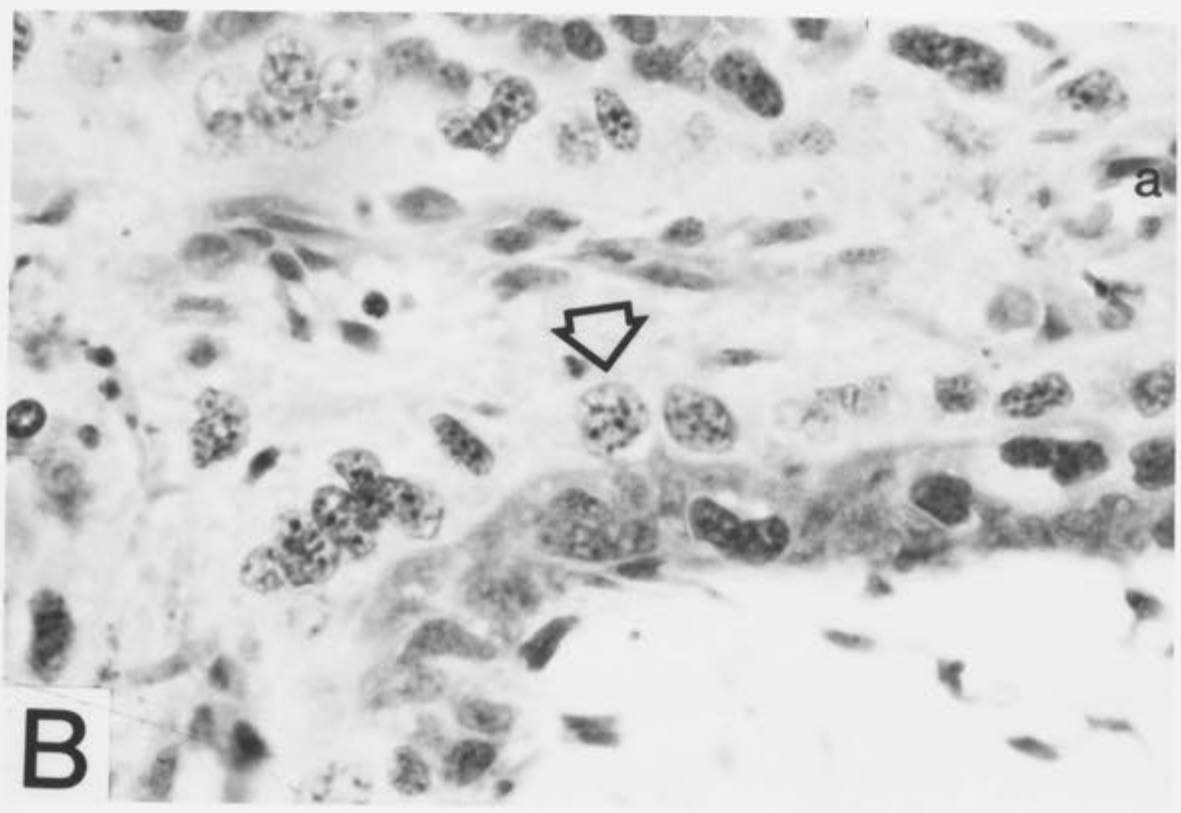
A: A placentome from a ewe at 49 d gestation. Nuclei of the syncytium are arrowed. Haematoxylin and eosin, x 400.

B: A placentome from a ewe at 60 d gestation. Nuclei of the syncytium are arrowed. a = maternal blood vessel. Haematoxylin and eosin, x 400.

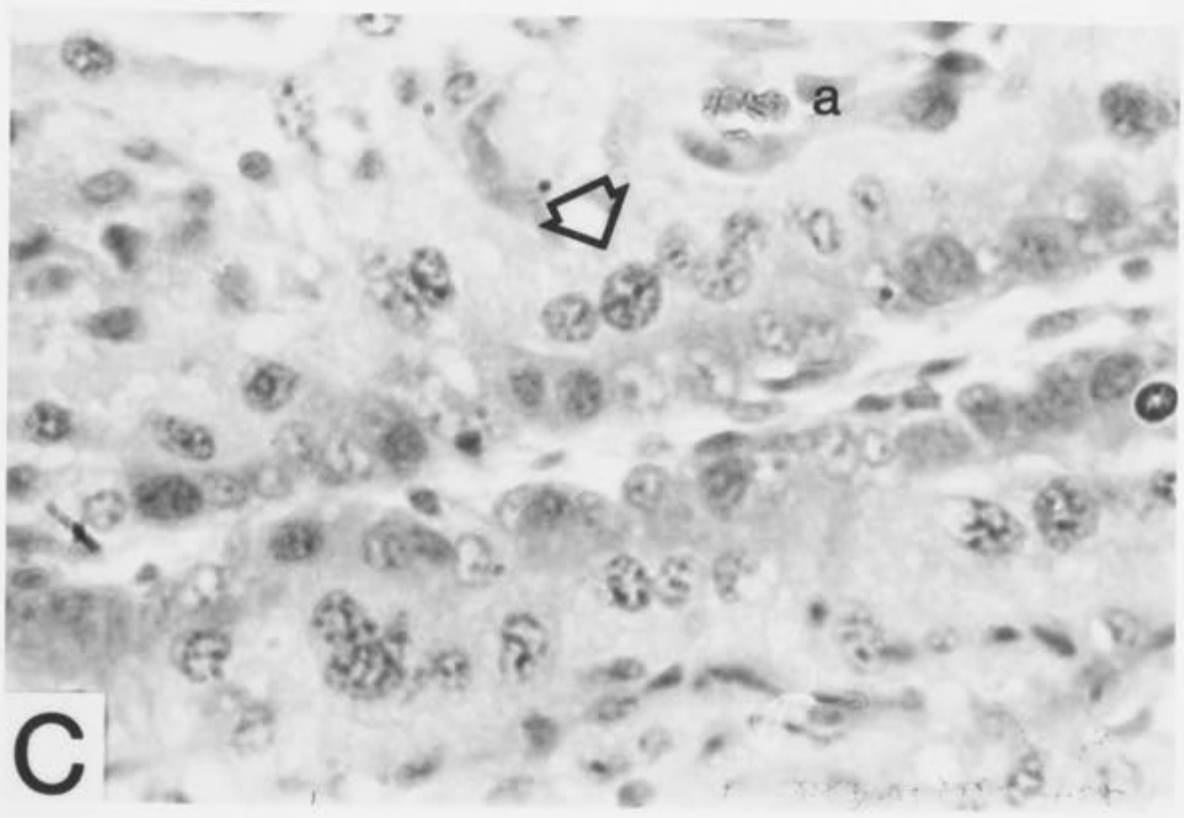
C: A placentome from a ewe at 76 d gestation. Nuclei of the syncytium are arrowed. a = maternal blood vessel. Haematoxylin and eosin, x 400.



A



B



C

with progress of gestation might be expected to facilitate such transmission. Clearly, morphological examination cannot give any indication of the capacity of placental cells to sustain virus growth at different gestational ages if this, rather than simple transit was a pre-requisite for secondary foetal infection.

Akabane virus, being an obligate intra-cellular parasite, can only infect the foetus as a result of its uptake by foetal cells. This could entail uptake of infective virions, or perhaps of infected cells such as the extravasated maternal blood cells which the trophoblast can ingest (Steven, 1975). Although the virus cannot replicate in uterine fluids, it probably survives for short periods. As it is heat labile with a half-life at 37^{o}C of about four hours (Parsonson and Della-Porta, personal communication) it is unlikely to survive for long periods. The probability of the virus invading foetal cells thus becomes greater in the post-implantation period, when cell to cell apposition is closer. Thereafter the chance of foetal infection should vary only if differences in hormonal levels or function of trophoblast occur. While the occurrence of pre-implantation infection of the foetus cannot be excluded, it seems less likely. The results of Parsonson et al (1981d) suggest that intrauterine inoculation of Akabane virus at the time of fertilization failed to cause either bovine embryonic infection or early embryonic mortality. Attempts at transplacental infection of the older but still pre-implantation embryo should assist in defining the influence of the placentome on foetal infection.

In conclusion, on morphological grounds it should be possible for Akabane virus to be transmitted across the placenta at any time after implantation, that is after 29 days. It might also be possible for maternal virus to spread to the pre-implantation embryo, and this question will be addressed in Chapter 5.

CHAPTER 2

THE *IN VITRO* RESPONSE OF FOETAL CELLS TO ALLOGENEIC CHALLENGE

CHAPTER 2 - THE IN VITRO RESPONSE OF FOETAL CELLS TO ALLOGENEIC CELLS

2.1 INTRODUCTION

Given the limitations that arose in attempting to use whole embryo culture to investigate the developing immune system, attention was directed to examination of the embryo and foetus *in situ*. The first foreign antigen to which responses were tested was allogeneic lymphocytes collected from efferent lymphatic vessels. These cells appeared to be a most appropriate stimulus as maternal leukocytes are likely to be the first 'non-self' determinants to which the foetus is normally exposed. Two methods were used to examine the response of the foetus to allogeneic lymphocytes. In the first, allogeneic cells were injected I/P into three foetuses *in utero* at 36 days gestation. As all of these foetuses were found to be dead when removed from the uterus four to seven days later, this approach was abandoned and replaced by the I/V injection of allogeneic cells into foetuses 2.5 h before their removal from the uterus. To determine the effect of foetal exposure to allogeneic lymphocytes in this way, foetal tissues were taken for histology, for organ culture, and for *in vitro* assessment of immune cellular reactivity.

Three types of assay techniques, each measuring a different endpoint, are available for the assessment of *in vitro* reactivity of immunologically competent cells to specific or non-specific stimuli. The first type of assay detects antibody produced *in vitro* specifically in response to antigen or non-specifically in response to some factor stimulating the

B-lymphocytes (Zanders, Smith and Callard, 1981). This assay is generally considerd to be an indicator of B-cell function.

The second group of assays involves the measurement of *in vitro* cellular cytotoxic responses, these being taken as indicators of the *in vivo* effector function of T-lymphocytes. The assays measure the release of ^{51}Cr consequent upon lysis of labelled target cells, and may involve lymphocytes directed against viral antigens or major histo-compatability antigens (Zinkernagel and Doherty, 1974; Lightbody, Bernoco, Miggiano and Cepellini, 1971).

The final group of assays is based on the transformation of lymphocytes when exposed to antigens - the cells enlarge, increase their rate of synthesis of deoxyribonucleic acid (DNA), and divide, in response to specific or non-specific stimulation. This response has been observed in both T and B lymphocytes, and is considered to reflect the recognition phase of immune cell function *in vivo*. One variant of this assay is the mixed lymphocyte culture assay (MLC) (Bach and Hirschhorn, 1964) which is considered to be an *in vitro* equivalent of the afferent or recognition phase of the *in vivo* response of thymus-derived lymphocytes to foreign histo-compatibility antigens (Asantila and Toivanen, 1976). It involves the co-culture of lymphocytes from two genetically different individuals, one population having been inhibited from dividing thereby making the response unidirectional. The response of cells in the uninhibited population to the allogeneic lymphocytes is assessed by measuring the rate of DNA synthesis.

Mixed lymphocyte assay was used in this experiment to in-

vestigate the ontogeny of immunocompetence in the sheep foetus, by assessing the capacity of cells from foetal donors of a variety of gestational ages to recognize and be activated by alloantigens, and by determining whether this capacity could be modified by previous in vivo exposure to alloantigens.

In designing an experiment to study the development of cellular immunocompetence in the sheep foetus, account was taken of the fact that there is an insufficient volume of blood in the young foetus to permit separation of the quantities of lymphocytes required for assay. While blood lymphocytes have been detected by 40 days (Osburn, 1980), they may not be present in significant numbers until approximately 60 days (Al Salami, personal communication) and their concentration remains very low until late in gestation. Account was also taken of the fact that allogeneic lymphocytes extravasated from the maternal circulation are likely to be among the first non-self determinants encountered by the foetus, and should therefore be an appropriate antigen for testing the responsiveness of the foetal lamb.

It was anticipated that the selected foetal age range, namely 39 - 76 days, would span the transition to at least partial cellular immunocompetence. Liver cells from normal foetuses aged 39 - 76 days, and from foetuses aged 24 - 59 days exposed to allogeneic cells for 2.5 hours in utero, followed by 24 h in vitro, were assayed in MLC with adult peripheral blood lymphocytes. Thymus becomes available in the older foetuses after approximately 50 days, and thymic lymphocytes from normal foetuses after this age were also used in mixed lymphocyte assays.

The aim of these experiments was both to examine the responsiveness of cells from young foetuses to allogeneic cells, and to test the stimulatory capacity of similar foetal cells for allogeneic lymphocytes from adult donors. To probe not only the capacity of cells from normal foetuses to respond on first exposure to allogeneic cells, but also the possibility that their responsiveness could be influenced by previous allogeneic stimuli, some foetuses were exposed to allogeneic lymphocytes before harvesting of their cells for testing. Because of the inability to culture the whole foetus *in vitro* for five days, this exposure was achieved by inoculation *in utero*. After allowing time for allogeneic lymphocytes to localize in various organs, liver was cultivated for 22 h before the cells were harvested for MLC, and liver and thymus were isolated and cultivated *in vitro* as organ fregments for periods of up to a week. It was reasoned that some, at least, of the processes of interaction between foreign lymphocytes and host cells might continue in culture.

2.2 EXPERIMENTAL DESIGN

Detailed techniques are described in Materials and Methods. Allogeneic lymphocytes to be injected into foetuses were collected from adult efferent lymph from a popliteal lymph node. The allogeneic lymphocytes used in MLC were adult peripheral blood lymphocytes.

Sixteen pregnant sheep and their 18 foetuses were divided into three groups that were treated as follows:

Group 1: Five foetuses aged 29 - 50 days were inoculated 2.5 h before recovery with 4.0×10^8 allogeneic adult efferent

lymphocytes which had been labelled with methyl ^{3}H-thymidine. Autoradiographs were prepared from the tissues of these foetuses to determine the distribution of the injected lymphocytes. It was necessary to determine in which foetal tissues the lymphocytes lodged before experiments were performed using foetuses injected with allogeneic lymphocytes, both to provide information about which organs could have been stimulated, and also to indicate the likelihood of "passaged" allogeneic lymphocytes being present in culture. This assisted in the selection of appropriate organs for examination, and the interpretation of the results of histological and immunological studies.

Group 2: Eight foetuses were recovered from seven untreated ewes between 39 and 76 days and suspensions of liver cells prepared from them were cultured for 24 hours. They were then assayed in MLC with adult peripheral blood lymphocytes for responsive, and in two cases stimulatory, capacity. Thymus cells from three of these foetuses (71 - 76 days) were similarly assayed. Fragments of liver (and thymus) from four (three) 40 to 50 day foetuses were cultured for seven days, and then fixed for histological examination. Uncultured liver and thymus were also fixed for histology. These foetuses provided control data for comparison with data from the challenged foetuses.

Group 3: Five foetuses aged from 24 to 59 days were challenged with allogeneic cells. They were injected I/V with 4.0×10^{8} allogeneic adult efferent lymphocytes 2.5 h before recovery. Liver cells from five of these foetuses

were cultured for 24 h , then assayed in MLC with adult peripheral blood lymphocytes for both responsive and stimulatory capacity. The donors of blood lymphocytes were not the donors of the efferent lymphocytes. Liver and sometimes thymus from seven of these 34 - 59 days lambs were fixed for histology. additionally liver and thymus fragments which had been cultured for seven days were then fixed.

2.3 RESULTS

Labelled allogeneic lymphocytes were detected principally in the liver after I/V injection into the foetuses of Group 1. A few labelled cells were detected in the spleen in some foetuses, but none was observed in other organs.

The responses to cultivation with allogeneic adult lymphocytes of cell suspensions prepared from foetal liver and thymus of untreated foetuses of 39 - 76 days gestation (Group 2 foetuses) are summarized in Table 2.1. There was a wide variation in the thymidine incorporation (counts per minute) of cells from different individuals cultured in the absence of other cells. However, comparison of stimulation index (SI) values, revealed a significant difference ($p<0.02$) between the rate of DNA synthesis of liver cells in response to allogeneic lymphocytes and the rate of DNA synthesis of the unstimulated liver cells. This finding suggests that some of the foetuses of 39 to 76 days may recognize and respond to alloantigenic stimulation. Since the only foetal donors to have SI of two or more were aged 49 and 76 days, cellular responsiveness may increase with foetal age. Whilst there were insufficient thymocyte assays to permit statistical analysis, cells from only one of the three foetuses, one aged 76 days,

TABLE 2.1 - Response of normal foetal cells to allogeneic adult lymphocytes

Tissue	Foetus	Foetal Age(d)	Response to PBL Cpm	SI	Response to autologous cells(FC) Cpm
Liver	82	39	863	1.9	451
	173	41	3451	1.6	2113
	94	47	891	1.7	516
	53	49	2482	3.7	671
	83	60	1530	1.5	1039
	102	71	509	0.8	680
	88 T1	76	1083	2.5	439
	T2	76	1964	2.0	990
Thymus	102	71	6427	0.9	6894
	88 T1	76	4293	2.0	1848
	T2	76	630	1.0	732

Cells in this and the succeeding three tables were cultured for three days, and were labelled with ^{3}H-thymidine 18 h before harvest.

PBL = allogeneic adult lymphocytes employed as the in vitro stimulus. All PBL were donated by the same individual.

Cpm = counts per minute of β emission from incorporated ^{3}H-thymidine.

SI = stimulation index = $\frac{\text{Cpm of PBL-stimulated cultures}}{\text{Cpm of self(FC)-stimulated cultures}}$

The response to autologous cells in this and the following three tables represents the outcome of culturing untreated cells with an equal number of irradiated autologous cells.

TABLE 2.2 - Response of normal adult lymphocytes to normal allogeneic foetal liver cells.

Tissue	Foetus	Age (d)	Response to FC Cpm	Response to FC SI	Response to PBL Cpm
Liver	173	41	13691	5.9	2320
	53	49	16949	2.9	5873

PBL = autologous adult lymphocytes

FC = foetal liver cells employed as the *in vitro* stimulus.

Cpm = counts per minute of β emission from incorporated ^{3}H-thymidine.

SI = stimulation index = $\frac{\text{Cpm of FC-stimulated cultures}}{\text{Cpm of self(PBL)-stimulated cultures}}$

TABLE 2.3 - Response of liver cells from foetuses previously exposed to allogeneic efferent lymphocytes (EL) to allogeneic lymphocytes (PBL).

Tissue	Foetus	Age (d)	Response to PBL		Response to autologous cells (FC)
			Cpm	SI	Cpm
Liver	213	31	13413	2.8	4799
	832	34	879	2.2	402
	932	39	28951	1.8	16023
	531	49	3586	2.8	1277
	123	59	3328	2.9	1144

EL = allogeneic adult efferent lymphocytes with which the foetuses were challenged in vivo.

PBL = allogeneic adult lymphocytes employed as the in vitro stimulus. EL and PBL were collected from different donors. All PBL were donated by the same individual.

Cpm = counts per minute of β emission from incorporated ^{3}H-thymidine.

SI = stimulation index = $\frac{\text{Cpm of PBL-stimulated cultures}}{\text{Cpm of self(FC) -stimulated cultures}}$

responded in MLC.

The response to stimulation by specific allogeneic lymphocytes of liver cells from foetuses inoculated with adult efferent lymphocytes 2.5 h before the foetuses were recovered are shown in Table 2.3. Here too there was a significant increase in the rate of DNA synthesis by liver cells after exposure to allogeneic lymphocytes ($p<0.001$). The response of cells from pretreated animals did not show any evidence of suppression: This is seen when the mean Cpm (10031) and SI (2.5) of the pretreated group is compared with the mean Cpm (1597) and SI (2.0) of the untreated group. The standard indices of the pretreated group were consistently higher than those of the untreated group (Table 2.1). This may be due to interaction between the efferent lymphocytes still present in the liver and the peripheral blood lymphocytes added to the culture as stimulators. Alternatively, it could be an effect of "precocious maturation" of foetal liver cells induced during the period following exposure to the efferent lymphocytes.

The capacity of liver cells from previously untreated foetuses to stimulate allogeneic cells in MLC was also assayed. These results (Table 2.2) revealed an increase in the DNA synthesis of adult lymphocytes cultured with foetal liver cells compared with the DNA synthesis of unstimulated lymphocytes. This suggests that foetal liver cells of 41 to 49 days may be capable of stimulating allogeneic lymphocytes. However, only two foetuses were thus assayed. In a similar manner, the capacity of liver cells from foetuses previously inoculated with allogeneic lymphocytes to stimulate allogeneic lymphocytes from a different donor was assayed in MLC.

TABLE 2.4 - Response of adult lymphocytes (PBL) to liver cells (FC) from foetuses previously exposed to allogeneic efferent lymphocytes (EL).

Tissue	Foetus	Age (d)	Response to FC		Response to autologous cells
			Cpm	SI	Cpm
Liver	213	31	1980	0.7	2870
	832	34	563	1.1	519
	932	39	23492	1.4	17104
	531	49	3846	0.7	5873
	123	59	7401	1.3	5739

EL = allogeneic adult efferent lymphocytes with which the foetuses were challenged in vivo.

PBL = autologous adult lymphocytes. EL and PBL were collected from different donors.

FC = foetal liver cells employed as the in vitro stimulus.

Cpm = counts per minute of β emission from incorporated ^{3}H-thymidine.

SI = stimulation index = $\frac{\text{Cpm of FC-stimulated cultures}}{\text{Cpm of self(PBL)-stimulated cultures}}$

TABLE 2.5 - Histological lesions in organs from untreated foetuses and from foetuses injected with allogeneic lymphocytes.

Foetus	Age(d)	Preculture liver	Postculture liver	Preculture thymus	Postculture thymus
Untreated					
173	41	-	-	ND	ND
94	47	-	-	-	-
53	49	-	-	-	-
141	50	-	-	-	-
Injected					
832	34	+	++	ND	ND
932	39	+	+	ND	ND
1511	40	+	++	ND	ND
993	40	+	-	ND	ND
531	49	+	+	ND	ND
1411	50	+	++	-	-
123	59		++	-	-

Foetal liver and thymus were fixed for histological examination at autopsy, and after their culture *in vitro* as organ fragments for seven days. Two sections of each specimen were thoroughly examined, as a result of which each specimen was scored on the following system:

(-) = normal

(+) = some necrosis of foetal cells present

(++) = foetal specimen totally necrotic

ND = not done.

MLC in which liver cells from the five injected foetuses of Group 3 were utilized as stimulators did not result in any stimulation of the allogeneic cells (Table 2.4). The results of the "reverse MLC" test using cells from untreated or inoculated foetuses as the stimulators do not support the possibility that the foetus contains cells capable of inhibiting mixed lymphocyte interactions.

Results of histological examination of foetal organs are shown in Table 2.5. Precultivation samples of liver from all of the inoculated foetuses were abnormal in comparison with liver samples from the control, uninoculated foetuses. Abnormalities detected in livers from inoculated foetuses included necrosis of parenchymal and haemopoietic cells, disruption of parenchymal architecture and increase in cell debris in sinusoids. The thymus from inoculated lambs appeared normal before cultivation. After seven days culture, liver from six of the seven inoculated foetuses showed more generalized cell necrosis than did the control cultures from untreated foetuses. Four of these livers from inoculated foetuses were totally necrotic. In contrast, cultured thymus from the two inoculated foetuses did not differ from the cultured thymus of the two equivalent controls. That is, injection of allogeneic lymphocytes caused no apparent damage to the thymus, either immediately or after organ cultivation for seven days. It did, however, appear to cause immediate liver damage within 2.5 hours of injection as evidenced by preculture samples, and severe or total necrosis of cultivated liver by seven days after injection. If allogeneic lymphocytes were to damage foetal tissues, those injected via the umbilical vein might be

expected to cause most damage in liver, as this is the first tissue that would be exposed.

2.4 DISCUSSION

There are several possible reasons for the deaths of the three foetuses inoculated at 36 days and left *in utero*. These include surgical trauma, susceptibility to Halothane, and action of the viable injected cells (for example graft-versus-host disease). A high rate of foetal death had also been observed in other studies in which foetal lambs of 50 - 70 days were inoculated with allogeneic cells from lymphoid organs (Mitchell, 1959; Moore and Rowson, 1961; Miyasaka, 1981), although the cause was not identified. From reports of experiments in which foetal lambs of this age were inoculated with conventional antigens (Silverstein *et al*, 1970; Fahey, 1977), there does not appear to have been a high rate of foetal death. If this was the case, it would support a graft-versus-host reaction as the cause of death following injection of allogeneic lymphocytes.

The liver is the major site of haemopoiesis in the young foetus (Asma, Pichler, Schuitt, Knapp and Hijmans, 1977), and is known to possess some lymphocyte precursors (Owen, Cooper and Raff, 1974; Phillips and Melchers, 1976). It is the only tissue likely to be involved in the immune response that is readily available in the early foetus. However, the proportion of the recovered liver cells used in the *in vitro* assays which comprised lymphoid cells is unknown. Furthermore, it is quite likely that this varied with the age of the individual foetus.

Since the mixed lymphocyte culture assay measures recognition of alloantigens by foetal cells, caution should also be exercised in comparing results of assays of cells from different donors. This is particularly so in this experiment, as the donors were derived from an outbred population and would be expected to vary considerably in their genetic compostion. Certainly, their level of unstimulated DNA synthesis varied greatly. Adult allogeneic lymphocytes in all experiments were taken from the same individual in an attempt to reduce genetic variability. Given these causes of variation, interpretation of the results would have been more satisfactory had there been a larger number of foetuses involved.

Interpretation is also limited by the fact that, while it is the best available, this is an *in vitro* assay, and its *in vivo* significance can only be inferred. It is possible that an assay system using disrupted organs exposes cells to antigens never encountered *in vivo*. Alternatively, removal from the normal environmental conditions present within an organ or an organism may result in cell behaviour unrelated to normal *in vivo* behaviour. Thus, a positive *in vitro* result should not be considered to be unequivocal evidence for a role of cell mediated immunity in protective immunity.

However, if the *in vitro* response does reflect an *in vivo* capacity, the above experiment implied that cells of foetuses as young as 41 days carry alloantigens which will stimulate allogeneic lymphocytes, and that foetal cells as young as 39 days can proliferate in response to some stimulus provided by

MLC. That is, they may have already acquired some degree of immunocompetence. However, this study did not discriminate between antigenically specific and non-specific responses. Of relevance to this distinction, Asantila and Toivanen (1976) suggested that early ovine foetal liver cells can respond non-specifically to blastogenic factors released by the T cells present in the stimulating cell population, rather than to alloantigens. Further, while the MLC is thought to reflect T cell reactivity, thymic lymphocytes have not been detected in foetuses younger than 50 days (Asantila and Toivanen, 1976; Al Salami, personal communication). Al Salami was unable to identify lymphocytes in the liver at any age, using electron microscopical methods. This would suggest that if the liver cells responding in MLC are lymphoid, they may be lymphocyte precursors, and are probably uncommitted or only partially committed.

In the case of pretreated foetuses, there is an alternative interpretation, namely that the responding cells in Table 2.3 were surviving injected allogeneic efferent lymphocytes rather than foetal cells. The poor stimulation reflected in Table 2.4 then becomes explicable by the fact that efferent lymphocytes are poor stimulators (Miyasaka, 1981). However, this interpretation does not seem likely in view of the observed stimulatory and responsive activity of liver cells from untreated foetuses.

The present study also demonstrated that the livers of foetuses receiving injections of allogeneic lymphocytes are rapidly damaged. This is presumably due to the presence of

the injected cells as most of these appeared to lodge in the liver. While this necrosis may be due to the mechanical trauma of the added cells, the total necrosis of cultured organ fragments suggest that the competent allogeneic cells may have recognized the foetal cells as foreign and therefore destroyed them.

The tendency of cells from foetuses inoculated with allogeneic lymphocytes to display higher rates of thymidine incorporation than those from control foetuses also suggest that the competent injected cells may be stimulated by foetal antigens in vivo. Similarly, the results of reverse MLC indicate that the liver cells of normal foetuses of 41 to 49 days are antigenic for allogeneic lymphocytes. In contrast, however, liver cells from foetuses previously exposed to allogeneic lymphocytes did not stimulate allogeneic lymphocytes in this in vitro assay. While there is no evidence, it is possible to speculate upon the reasons for this apparent suppression of stimulatory activity. It may be a suppression of function only, or, in view of the liver necrosis which followed injection of allogeneic lymphocytes, there may have been necrosis of the potentially stimulatory foetal cells.

In conclusion, cells recoverable from the livers or normal foetuses as young as 39 days, and from foetuses previously exposed to alloantigens as early as 31 days, are capable of transforming, whether specifically or non-specifically, in response to culture with allogeneic lymphocytes. This may imply the presence of some degree of immunocompetence even at this very early stage. Competent allogeneic lymphocytes injected I/V into the foetus appeared to localize in the liver, where they caused necrosis of foetal tissue.

CHAPTER 3

THE RESPONSE OF THE ADULT EWE TO AKABANE VIRUS

CHAPTER 3 - THE RESPONSE OF THE ADULT EWE TO AKABANE VIRUS

3.1 INTRODUCTION

The response of the fully competent adult ewe was examined to provide a baseline for comparison with the response of foetal lambs. In examining this response, several of the various tests of immune function which had been used in other studies of the ontogeny of lymphoid function (Asantila *et al*, 1976; Leino, 1978; Robinson, 1983; Perryman, McGuire and Torbeck, 1980; Hall and Morris, 1962; Liggitt, De Martini and Pearson, 1982) were selected. Both humoral and cellular components of the responses were studied. Specific neutralizing antibody was selected as an indicator of humoral responsiveness. Measurement of specific neutralizing antibody was selected because it was the most likely parameter to be relevant to *in vivo* infection. It was undertaken on samples of sera and efferent lymph.

Lymphocyte incorporation of ^{3}H-thymidine was employed as an indicator of immune cell function. The capacity of adult peripheral blood lymphocytes and efferent lymphocytes to respond to *in vitro* stimulation was determined by the lymphocyte transformation assay (LTA) (Janossy and Greaves, 1971). The basis of this assay was the incorporation of ^{3}H-thymidine by lymphocytes involved in DNA synthesis. This DNA synthesis and increased protein synthesis accompanies the transformation of small resting lymphocytes into proliferating lymphoblasts by the selected stimuli. The mechanism by which this transformation occurs is thought to involve biochemical changes at the level of the cell membrane which are followed by events

associated with derepression of eukaryotic genomes (Douglas, 1971). The stimuli selected included non-specific activators or mitogens, such as the plant lectin Concanavalin A (Con A) and the specific antigens of Akabane virus. In both cases, the gross morphological and functional changes in stimulated lymphocytes are similar, but non-specific activators presumably by-pass any requirement for specific recognition that occurs in the activation characteristic of immune responses (Schechter et al, 1977). Consequently they stimulate a sizable proportion of the lymphocytes of normal individuals, without any requirement for presensitization of the lymphocyte donors (Waithe and Hirschhorn, 1978). A characteristic of the response to some activators is that it may be restricted to a single functionally defined cell type. For example, Con A is considered to be a T-cell activator (Janossy and Greaves, 1971).

Under some circumstances viruses are capable of non-specifically suppressing in vitro lymphocytic function. Some examples that have been reported are Avian retrovirus and African swine fever virus (Wainberg, Vydelingum and Margolese, 1983; Wardley, 1982). Although the relevance of in vitro transformation to the in vivo protective immune response is totally unknown and thus such cell assays can only be used with great caution to predict the effects in the whole animal, it was considered that the in vitro information would be valuable. In particular, it provided a means of testing for the possibility of non-specific suppression. This was examined in nine adult ewes by determining the effect of concurrent presence of viable or inactivated Akabane virus on the response

of efferent lymphocytes and peripheral blood lymphocytes to Con A. It was reasoned that any indication of viral suppression of the adult immune system would give some indication of whether a similar effect might be exerted on the immature system.

Lymphatic cannulations were performed on some of the ewes after which the draining lymph node was challenged. Lymph flow rate, cell output and cell type were determined for each collection to provide further sequential information on cellular immune reactivity at intervals after challenge with virus. Previous studies on the local response in ovine peripheral lymph nodes challenged with non-viable antigens such as those in human serum (Hall and Morris, 1962), and with the potentially replicating antigens Ross River virus and Kunjin virus (Pearson, Doherty, Hapel and Marshall, 1976), have in general produced a characteristic series of changes. After an initial decrease in cell output (Trnka and Cahill, 1980) there is an increase in total cell output commencing around 24 h after challenge followed by an increase in the proportion of efferent cells which are lymphoblasts.

The experimental design involved injection of Akabane virus into two groups of normal ewes either via the jugular vein, or subcutaneously in the region drained by the popliteal lymph node. Another group of control ewes was left untreated. To ascertain the effect of the virus on adult sheep, ewes were monitored for signs of clinical disease and tissues were taken from a number of them two weeks after infection for histopathological examination. Three aspects of the immunological

response to the virus were also examined, namely the production of serum antibody, virus induced changes in efferent lymph after local challenge, and *in vitro* transformation responses to mitogens and Akabane viral antigens.

3.2 EXPERIMENTAL DESIGN

Three groups of normal non-pregnant ewes were treated as follows:

Group 1: Fourteen ewes were inoculated via the jugular vein with 10^7 PFU of Akabane virus in ten ml Hanks' BSA. Peripheral blood lymphocytes (and in the case of ewe 17, popliteal efferent lymphocytes) were taken from these sheep at two to five day intervals for up to three weeks after inoculation. Lymphocyte transformation assays were performed using Con A, LPS, PHA and Akabane virus as activators. Transformation assays were also performed to detect any suppressive effect of the virus on *in vitro* lymphocyte function. The response of peripheral blood lymphocytes, and in the case of ewe #47, of efferent lymphocytes, were assayed before and after infection to the combined presence of Con A and Akabane virus. A number of tissues from three of these ewes were selected at autopsy for histological examination. Brain, spinal cord and skeletal muscle were examined because these tissues have been reported to be abnormal in foetal sheep affected by Akabane disease. Ovary was the only adult organ found to be abnormal in a study involving Akabane infection of adult cattle (Parsonson *et al*, 1981d) and was therefore also selected in this study. Spleen was examined because, being a central lymphoid organ, it might be expected to show some morphological changes reflecting the immunological response.

Group 2: Three ewes were inoculated S/C in the region drained by the popliteal lymph node with 10^7 PFU of Akabane virus in 1.0 ml of medium. Popliteal efferent lymphocytes, and in the case of ewe 93, peripheral blood lymphocytes also, were collected at two to five day intervals for up to two weeks after inoculation and assayed for response to Con A and to Akabane virus.

Group 3: A single collection of peripheral blood lymphocytes was taken from three control ewes and was assayed for response to Con A and to Akabane virus.

Samples from all groups were assayed for specific neutralizing antibody. If efferent lymphocytes were collected, records were kept of lymph volume and cell count of all samples. All animals were monitored for signs of clinical disease, namely changes in physical appearance, demeanour, appetite and rectal temperature.

3.3 RESULTS

No sheep gave any indication of clinical disease, nor were pathological changes detected in any of the three sheep examined. Specific neutralizing antibody appeared in efferent lymph of locally infected ewes five to six days after infection (Fig. 3.1). Neutralizing antibody was detected in the lymph of one of these (ewe 93) approximately three days before it was detected in the serum. Serum antibody levels of the other three cannulated ewes were not monitored. Antibody was detected in the efferent lymph of a systemically infected ewe three days after infection. The specific neutralizing anti-

Fig 3.1 Specific neutralizing antibody to Akabane virus in efferent lymph from adult popliteal lymph nodes, after challenge with Akabane virus.

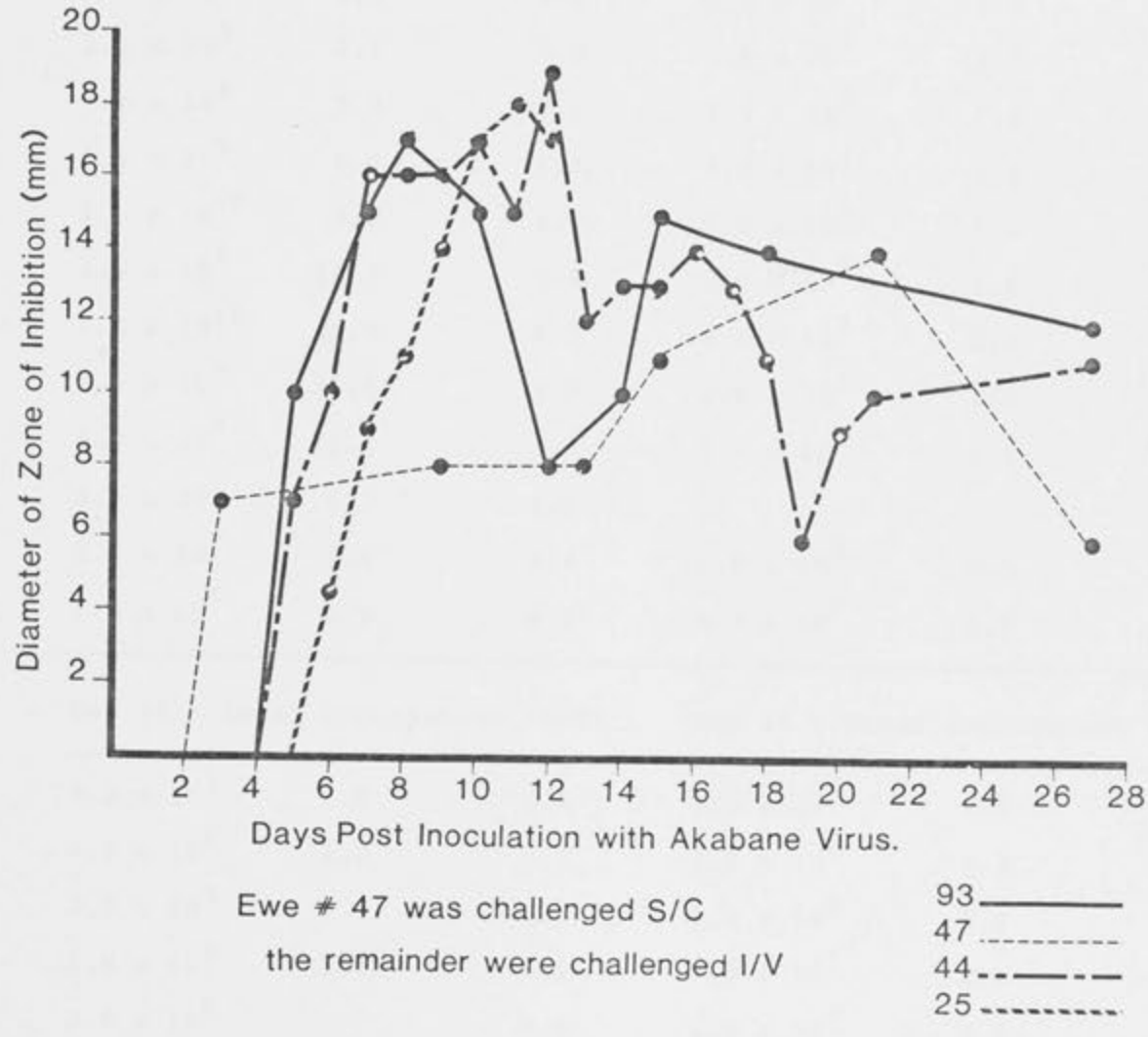


TABLE 3.1 - Efferent lymph data from ewes infected with 10^7 PFU of viable Akabane virus

Days post-inoculation	Ewe 93 - local inoculation (S/C)			Ewe 47 - systemic inoculation (I/V)		
	Total cell count/h	Percentage blast cells	Lymph flow rate (ml/h)	Total cell count/h	Percentage blast cells	Lymph flow rate (ml/h)
-1	1.6×10^7	1.1	2.4	3.4×10^6	1.0	3.3
0	3.2×10^7	1.5	5.0	3.8×10^7	1.3	4.2
1	2.3×10^6	2.7	6.0	1.8×10^7	1.9	4.2
2	1.1×10^9	2.1	8.3	1.9×10^7	1.7	3.7
3	7.4×10^9	2.3	7.1	1.7×10^7	1.0	4.2
4	1.6×10^9	6.0	6.4	3.0×10^7	1.2	4.8
5	1.1×10^{10}	9.0	5.4	3.9×10^7	1.9	4.6
6	1.5×10^8	14.7	3.9	3.6×10^7	2.6	3.9
7	1.8×10^{10}	19.0	4.5	4.5×10^7	2.2	4.3
8	1.4×10^9	16.8	5.0	3.8×10^7	4.0	3.4
9	5.2×10^7	12.4	3.3	3.6×10^7	2.5	3.2
10	4.5×10^7	6.3	4.9			
11	2.7×10^7	5.4	4.4	1.0×10^8	2.5	2.8
12	1.6×10^7	4.0	4.5	4.3×10^7	1.9	2.5
	Ewe 44 - local inoculation (S/C)			Ewe 25 - local inoculation (S/C)		
-1	5.3×10^7	1.0	5.5	1.1×10^7	2.1	3.3
1	4.6×10^6	1.2	5.1	6.0×10^6	1.3	3.0
2	2.0×10^8		5.0	1.0×10^8	2.2	3.0
3	1.8×10^8	2.7	5.1	7.0×10^7	2.6	4.8
4	3.0×10^8		5.6	1.0×10^8	6.0	6.0
5	2.8×10^8	9.5	5.9	1.6×10^8	9.2	5.6
6	7.9×10^7		5.8	1.3×10^8	10.4	3.3
7	2.8×10^8	11.4	4.6	1.4×10^8	13.5	2.3
8	2.0×10^8		4.0	1.3×10^8	12.7	2.1
9	1.5×10^8	7.3	3.5	5.0×10^7	8.6	2.8
10	1.5×10^7		4.5	2.8×10^7	5.6	2.0
11	6.3×10^7	6.1	3.5			

body levels in lymph of all of the locally challenged ewes followed a common pattern, rising sharply to peak at eight to 12 days post-inoculation. However, while specific neutralizing antibody appeared at three days in the lymph of the systemically-challenged ewe, it remained at its initial level until 13 days, when it rose to a peak at about 21 days before declining.

Rates of flow and cell output of efferent lymph collected from four infected ewes were measured (Table 3.1). The three locally inoculated ewes (93, 44 and 25) showed a slight rise in lymph flow rate between two and five days, while lymph flow rate in the two that had been systemically inoculated remained constant. Total cell count/h in lymph from the ewe infected systemically remained constant, as did the blast cell count. However, total cell count/h in lymph of the three ewes challenged locally showed an initial five to ten-fold drop on the first day after challenge, followed by a one or two log increase which peaked at five to seven days, and returned to pre-challenge levels by ten to 12 days as had occurred in previous studies of local lymph node responses. All three locally challenged ewes showed a significant increase in the blast cell proportion from three to four days (Table 3.1). Blast cell content peaked at six to seven days before declining. This substantial increase in the cellular output from the draining lymph node, accompanied by an increase in the frequency of blast cells in the population, was seen in response to local, but not systemic challenge.

The capacity of lymphocytes from infected and unchall-

TABLE 3.2 - In vitro response of peripheral blood lymphocytes from normal adult ewes to mitogen and antigen

Ewe	Infection status	Controls		Non-specific stimulation		Specific stimulation			
		Medium Cpm[a]	Vero Cpm	Con A Cpm	SI[b]	Akabane Cpm	SI	Inactive Akabane Cpm	SI
14	Not inoculated	744[c]	2612	8570	11.5	2340	0.9	75	0
42	" "	250	598	8279	33.1	656	1.1	831	1.4
99	" "	469	1176	2744	5.9	1015	0.9	42	0
8	Pre-inoculation	756	1381	81791	108.2				
22	" "	459	831	108862	237.2				
7	" "	722	761	88260	122.2				
811	" "	280	554	15570	55.6	188	0.3	282	0.5
13	" "	898	943	48546	54.1	260	0.3	554	0.6
19	" "	410	381	16252	39.6	208	0.5	160	0.4
251	" "	468	353	44966	96.1	245	0.7	346	1.0
47	" "	234	2623	9814	41.9	2233	0.9	1231	0.5

a Cpm = counts per minute of β emission from incorporated ^{3}H-thymidine.

b SI = stimulation index. SI for Con A = $\frac{\text{Cpm of cultures with Con A}}{\text{Cpm of cultures with medium only}}$

SI for virus = $\frac{\text{Cpm of cultures with virus}}{\text{Cpm of cultures with Vero}}$

In this and the succeeding seven tables, indices greater than 2.0 are considered to indicate the presence of a response.

C = In this and the succeeding seven tables, Cpm values are the mean of three replicates. Standard errors did not exceed 15% and so have been omitted from the tables for clarity.

TABLE 3.3.1 - In vitro response of lymphocytes of an adult ewe infected systemically with Akabane virus to mitogen and antigen

Ewe	Time(d) post-inoculation	Source of lymphocytes	Medium Cpm[a]	Vero Cpm	Con A Cpm	SI[b]	Akabane Cpm	Akabane SI	Inactivated Akabane Cpm	Inactivated Akabane SI
47	-1	A Efferent lymph	398	770	10854	27.3	363	0.5	557	0.7
	3	" "	2990	535	20653	6.9	835	1.6	519	1.0
	6	" "	400	404	17923	44.8	1066	2.6	601	1.5
	9	" "	881	431	24825	28.2	1819	4.2	931	2.2
	12	" "	646	923	49241	76.2	2845	3.1	779	0.8
	15	" "	621	498	16031	25.8	1225	2.5	307	0.6
	21	" "	574	282	24637	42.9	780	2.8	397	1.4
	28	" "	954	699	22997	24.1	849	1.2	204	0.3
	-1	B Peripheral blood	234	756	9814	41.9	1336	1.8	959	1.3
	3	" "	937	1947	23048	25.0	2312	1.2	2591	1.3
	6	" "	570	542	12664	22.2	1332	2.5	919	1.7
	9	" "	545	681	8495	15.6	3432	5.0	1212	1.8
	12	" "	564	1975	22108	39.2	5983	3.0	1462	0.7
	15	" "	337	510	10123	30.0	1967	3.9	807	1.6
	21	" "	579	1499	48323	83.5	3896	2.6	1862	1.2
	28	" "	954	699	22997	24.1	849	1.2	204	0.3

a Cpm = counts per minute of β emission from incorporated ^{3}H-thymidine.

b SI = stimulation index. SI for Con A = $\frac{\text{Cpm of cultures with Con A}}{\text{Cpm of cultures with medium only}}$

SI for virus = $\frac{\text{Cpm of cultures with virus}}{\text{Cpm of cultures with Vero}}$

TABLE 3.3.2 - In vitro response of peripheral blood lymphocytes from adult ewes infected systemically with Akabane virus to mitogens and antigen.

Ewe	Time(d) post-inoculation	Medium Cpm[a]	Vero Cpm	Con A Cpm	SI[b]	Akabane Cpm	Akabane SI	Inactive Akabane Cpm	Inactive Akabane SI	LPS Cpm	LPS SI	PHA Cpm	PHA SI
221	5	318	870	7245	22.8	1562	1.8			1850	5.8	1817	5.7
	14	689	6690	3898	5.7	8565	1.3	11943	1.8	182	0.3	2371	3.4
	20	1148	5069	32737	28.5	6291	1.2	7154	1.4	4695	4.1	7569	6.6
	25	164	146	3553	21.7	521	2.7	497	2.6			1065	6.5
27	4	717	715	34623	48.3	2247	3.1						
	10	89	503	1722	19.3	670	1.3	44	0.1				
	14	157	561	4765	30.4	1263	2.3	115	0.2				
	21	119	1222	3279	27.3	567	0.5	66	0.1				
36	4	4049	635	65383	16.1	10748	16.9[c]						
	10	1709	347	9566	5.5	5493	15.8[c]	990	2.8				
	14	2423	541	59028	24.4	3553	6.7[c]	52	0.1				
	21	1414	2304	4750	3.4	2786	1.2	32	0				
811	3	257	509	34069	132.6	650	1.3	183	0.4				
	6	1173	2038	41959	35.8	6454	3.2	320	0.2				
13	3	559	1069	58391	104.5	2612	2.4	224	0.5				
	6	1099	1338	64111	58.3	5186	3.9	623	0.5				
19	3	1273	1548	97274	76.4	987	0.6	387	0.3				
	6	1223	689	36644	30.0	4526	6.6	347	0.5				
251	3	703	344	113071	160.8	898	2.6	269	0.8				
	6	828	573	54319	65.6	2076	3.6	1063	1.9				

a Cpm = counts per minute of β emission from incorporated ^{3}H-thymidine.

b SI = stimulation index. SI for Con A = $\frac{\text{Cpm of cultures with Con A}}{\text{Cpm of cultures with medium only}}$

SI for virus = $\frac{\text{Cpm of cultures with virus}}{\text{Cpm of cultures with Vero}}$

c = These indices appear to have been distorted by the depressed Cpm values for culture with Vero.

TABLE 3.3.3 - In vitro response to Con A of peripheral blood lymphocytes of adult ewes infected systemically with Akabane virus.

Ewe	Time(d) post-inoculation	Medium Cpm[a]	Con A Cpm	SI[b]
31	2	345	2834	8.2
	4	559	7948	14.2
	9	938	64371	68.6
	12	1143	39826	34.8
	26	108	2922	27.0
32	7	141	656	4.7
	9	341	4419	13.0
	11	300	4491	15.0
	13	530	19891	37.5
	15	323	9224	28.6
26	2	255	673	2.6
	4	1902	51305	27.0
	9	1887	95321	50.5
	12	1682	67966	40.4
	26	248	6568	26.5
7	3	855	108065	126.4
	6	2059	202305	98.3
	11	721	29480	40.9
8	3	170	14067	82.7
	6	7111	78373	11.0
	11	1085	36158	33.3
221	3	336	4995	14.9
	6	402	23444	58.3
	11	250	5357	22.1

Cpm[a] = counts per minute of β emission from incorporated ^{3}H-thymidine

SI[b] = stimulation index = $\frac{\text{Cpm of cultures with Con A}}{\text{Cpm of cultures with medium alone.}}$

TABLE 3.4 - In vitro response of lymphocytes of adult ewes infected locally with Akabane virus to mitogen and antigen.

Ewe	Time post-inoculation(d)	Source of lymphocytes	Medium Cpm[a]	Vero Cpm	Con A Cpm	Con A SI[b]	Akabane Cpm	Akabane SI	Inactive Akabane Cpm	Inactive Akabane SI
25	-1	A Efferent lymph	1813	1309	115	0.1	1620	1.2	3347	2.6
	1	" "	4275	1522	7101	1.7	6647	4.4	5095	3.3
	3	" "	1515	1124	7204	4.6	2022	1.8	1420	1.3
	5	" "	7083	1108	10088	1.4	4071	3.7		
	7	" "	1121	440	580	0.5	559	1.3	197	0.4
	9	" "	6472	828	1755	0.3	588	0.7	386	0.5
	11	" "	683	932	545	0.8	1436	1.5	266	0.3
44	-1	" "	867	1091	651	0.8	2180	2.0	1390	1.3
	1	" "	858	371	643	0.7	486	1.3	4507	12.1
	3	" "	1285	1126	864	0.7	341	0.3	937	0.8
	5	" "	4518	2184	4917	0.4	3788	1.7	2527	1.2
	7	" "	1549	2114	10398	6.7	1813	0.9		
	9	" "	841	1879	6138	7.3	1823	1.0	2446	1.3
	13	" "	855	1621	52886	61.9	1343	0.8	969	0.6
	14	" "			35707	54.0				
	16	" "			31300	12.5				
	20	" "			2142	29.8				
	24	" "			11143	15.8				
	29	" "			12221	63.0				
93	-1	" "	633	509	32679	51.6	915	1.8	695	1.4
	2	" "	786	1150	10888	13.9	1424	1.2		
	6	" "	2940	18435	23589	11.6	15692	0.9	5691	0.3
	9	" "	1110	17236	21044	19.0	14840	0.9	3210	0.2
	15	" "	870	776	38764	44.6	4997	6.4		
	2	B Peripheral blood	1035	708	1260	1.2	1519	2.1		
	6	" "	2940	2388	20688	7.0	4249	1.8		
	9	" "	2714	5346	35022	12.9	9538	1.8	4422	0.8

a Cpm = counts per minute of β emission from incorporated ^{3}H-thymidine.

b SI = stimulation index. SI for Con A = $\frac{\text{Cpm of cultures with Con A}}{\text{Cpm of cultures with medium only}}$

SI for virus = $\frac{\text{Cpm of cultures with virus}}{\text{Cpm of cultures with Vero}}$

enged ewes to respond to virus was compared (Tables 3.2, 3.3 and 3.4). In each instance, the response to both live and inactivated virus was measured. As will be seen from the tables, in all cases the response to inactivated Akabane virus was less than that to viable virus. The results reported in the following text refer to stimulation by viable virus.

The response to viral antigen of efferent lymphocytes collected from the three ewes before local challenge with virus (Table 3.4, A) did not exceed their response to Vero cells alone, indicating that there was no net stimulation by Akabane virus. Efferent lymphocytes collected from the node after local challenge (Table 3.4,A, Fig. 3.2) gave inconsistent net responses to virus in two individuals, and no net response in the third. Peripheral blood lymphocytes from one of these responding ewes were also tested, and gave a marginal net response on one of the three days (Table 3.4, B).

The response of peripheral blood lymphocytes from control ewes to Akabane virus (Table 3.2), did not exceed that to Vero cells. Peripheral blood lymphocytes from all eight ewes infected systemically (Tables 3.3.1,B, 3.3.2, Fig. 3.3) gave a response that was significantly greater than that of control ewes ($p < 0.01$). The response rose with time after infection to peak at seven to 12 days before decreasing (Fig. 3.4). Efferent lymphocytes from one of these ewes (#47) were also tested, with similar results (Table 3.3.1, A). There was a net response to virus which differed significantly from the pre-inoculation response ($p < 0.001$) and which rose with time after infection to peak at approximately nine days before decreasing to pre-infection levels by 21 days.

Fig 3.2 Response to Con A of efferent lymphocytes from adult ewes before and after infection with Akabane virus.

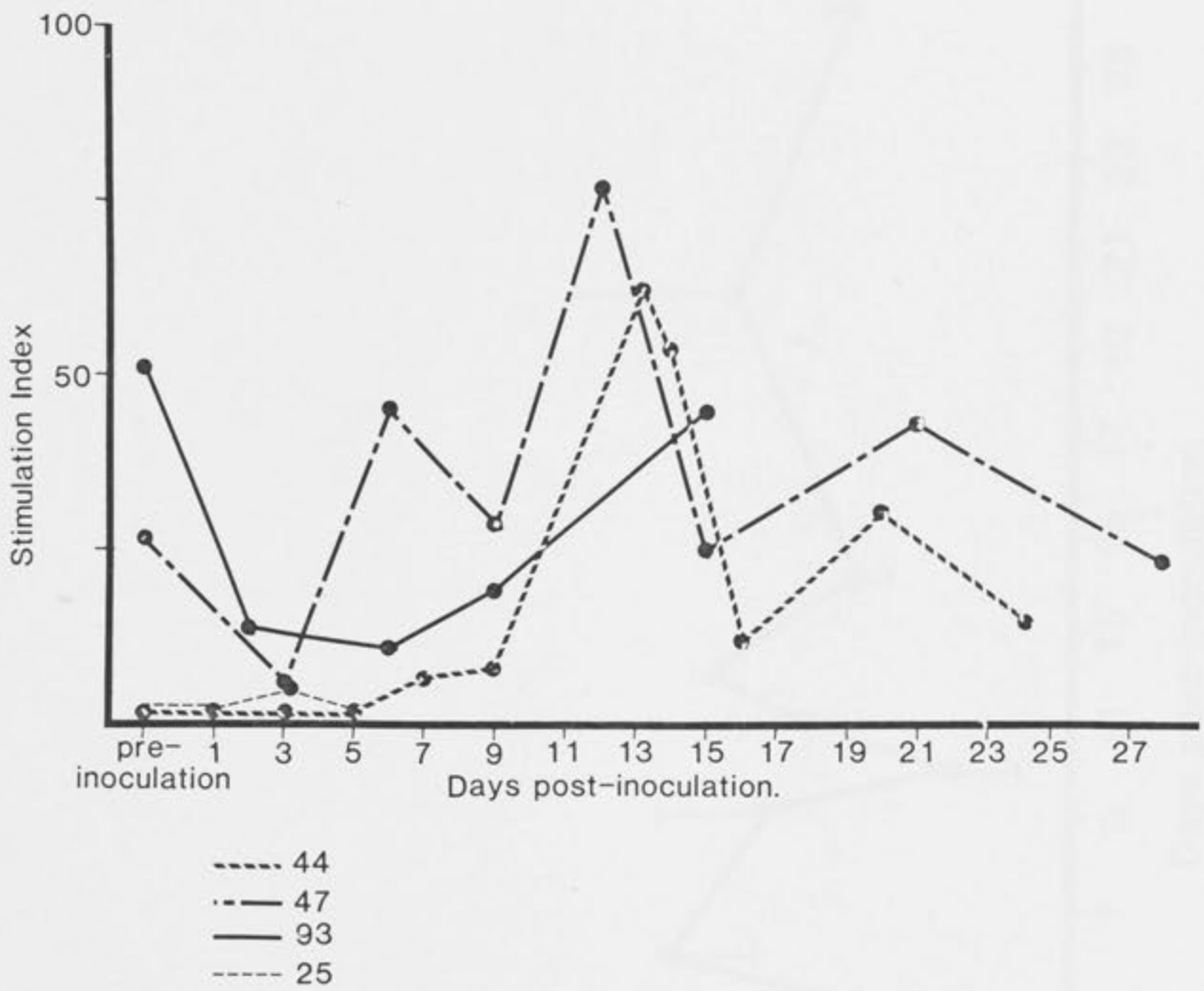
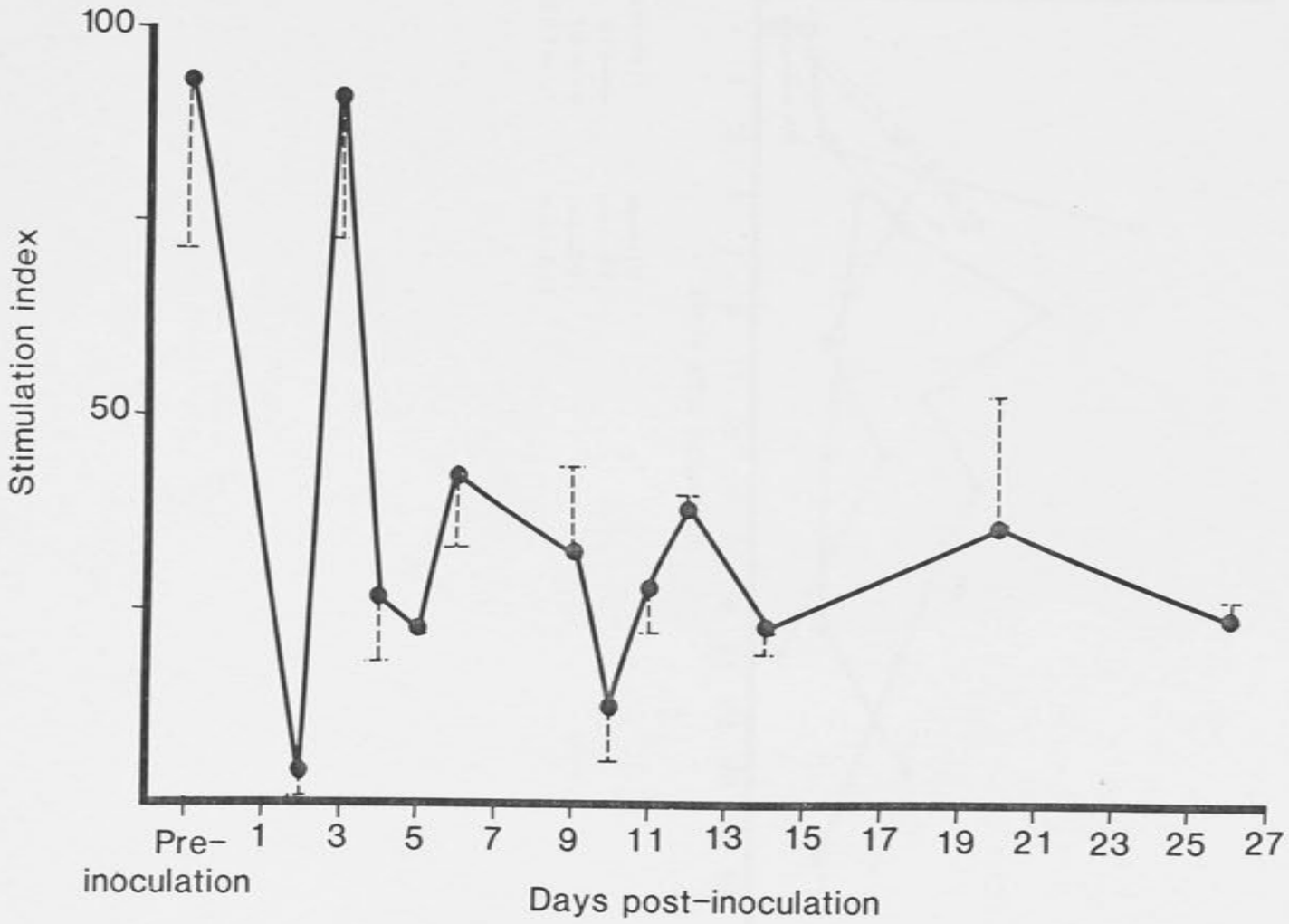
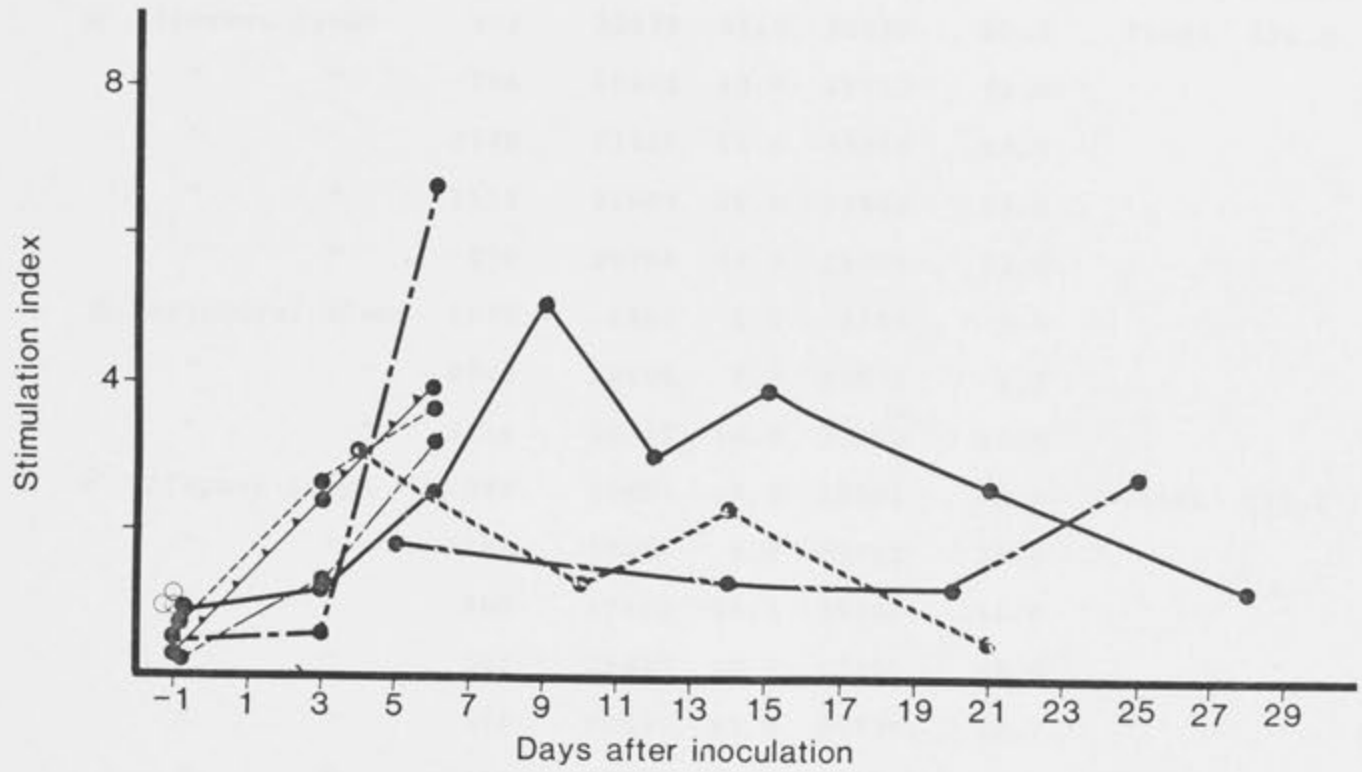


Fig 3.3 Response to Con A of peripheral blood lymphocytes from adult ewes before and after infection with Akabane virus.



The values plotted are the means of the stimulation indices obtained from all ewes whose blood lymphocytes were assayed.

Fig. 3.4. Response to Akabane virus of peripheral blood lymphocytes from adult ewes before and after systemic infection with Akabane virus.



controls ○ ▲—▲ 13
47 —— ----- 27
19 — - - ——— 221
251 ----- - — 811

TABLE 3.5.1 - *In vitro* response of adult lymphocytes to combined stimulation of Con A and Akabane virus before and after *in vivo* infection with Akabane virus.

Ewe	Time post-inoculation	Source of lymphocytes	Medium Cpm[a]	Con A Cpm	SI[b]	Con A + Akabane Cpm	Con A + Akabane SI	Con A + Vero Cpm	Con A + Vero SI
93	-1	A Efferent lymph	633	32679	51.6	38922	61.5	72193	114.0
	2	" "	786	10888	13.9	48732	62.0		
	6	" "	2940	23589	11.6	57918	19.7		
	9	" "	1110	21044	19.0	53946	48.6		
	15	" "	870	38764	44.6	29406	33.8		
	2	B Peripheral blood	1035	1260	1.2	3830	3.7		
	6	" "	2940	20688	7.0	25578	8.7		
	9	" "	2714	35022	12.9	35011	12.9		
47	-1	A Efferent lymph	398	10854	27.3	27701	69.6	44614	112.1
	3	" "	2990	20653	6.9	83720	28.0		
	6	" "	400	17923	44.8	58760	146.9		
	9	" "	881	24825	28.2	43081	48.9		
	21	" "	574	24637	42.9	35739	62.7		
	28	" "	954	22997	24.1	43407	45.5		
	-1	B Peripheral blood	234	9814	41.9	19180	82.0	40271	172.1
	3	" "	937	23048	25.0	24643	26.3		
	6	" "	570	12664	22.2	7296	12.8		
	9	" "	545	8495	15.6	5232	9.6		
	21	" "	579	48323	83.5	33756	58.3		

Ewe 93 was infected S/C. Ewe 47 was infected I/V.

Stimulation by Con A plus Vero antigen is included as a control.

a Cpm = counts per minute of β emission from incorporated ^{3}H-thymidine.

b SI = stimulation index = $\frac{\text{Cpm of cultures with Con A}}{\text{Cpm of cultures with medium alone}}$

TABLE 3.5.2 - *In vitro* response of adult PBL to combined stimulation by Con A plus Akabane virus, before and after *in vivo* infection with Akabane virus

Ewe	Time(d) post-inoculation	Medium Cpm[a]	Con A Cpm	SI[b]	Con A + Akabane Cpm	Con A + Akabane SI	Con A + Vero Cpm	Con A + Vero SI
7	-1	722	88260	122.2	46565	64.5	78598	108.9
	3	855	108065	126.4	79460	92.9	88817	103.9
	6	2059	202305	98.3	73714	35.8	157843	76.7
	11	721	29480	40.9	76329	93.4	64056	88.8
8	-1	756	81791	108.2	39858	52.7	45867	60.7
	3	170	14067	82.7	10396	61.2	7808	45.9
	6	7111	78373	11.0	46715	6.6	79606	11.2
	11	1085	36158	33.3	27589	25.4	45445	41.9
22	-1	459	108862	237.2	13421	29.2	52346	114.0
	3	336	4995	14.9	15846	47.2	16052	47.8
	6	402	23444	58.3	7526	18.7	53847	133.9
	11	250	5357	22.1	31250	125.0	3841	15.4
811	-1	280	15570	55.6	17116	61.1	12071	43.1
	3	257	34069	132.6	18662	72.6	22319	86.8
	6	1173	41959	35.8	33357	28.4	41919	39.2
13	-1	898	48546	54.1	41887	46.6	56181	62.6
	3	559	58391	104.5	56974	101.9	53816	96.3
	6	1099	64111	58.3	64625	58.8	58010	52.8
19	-1	410	16252	39.6	44196	107.7	21496	52.4
	3	1273	97274	76.4	84499	66.4	83980	66.0
	6	1223	36644	30.0	47675	39.0	38187	31.2
251	-1	468	44966	96.1	52788	112.6	52338	111.8
	3	703	143071	160.8	83430	118.7	99837	142.0
	6	828	54319	65.6	1505	2.6	26419	31.9

The ewes were infected I/V. Stimulation by Con A plus Vero antigen is included as a control.

a Cpm = counts per minute of β emission from incorporated ^{3}H-thymidine.

b SI = stimulation index = $\frac{\text{Cpm of cultures with Con A}}{\text{Cpm of cultures with medium only}}$

The responsiveness to Con A of lymphocytes from challenged and unchallenged ewes was compared (Tables 3.2, 3.3, and 3.4). The peripheral blood lymphocytes of control ewes (Table 3.2) did respond to Con A, with this response varying between individuals. Peripheral blood lymphocytes from ewes infected by I/V or S/C routes attained a significantly higher peak response ($p<0.01$) than did the peripheral blood lymphocytes of control ewes (Tables 3.3.1,B, 3.3.2, 3.3.3, 3.4,B). This response varied with time after inoculation (Fig. 3.3). In general, responsiveness rose initially, to be followed by a period of diminished response at five to nine days after inoculation, and then by a slight rise.

The response (Tables 3.3.1,A, 3.4, 3.4,A) to Con A of efferent lymphocytes of ewes infected locally or systemically was depressed for the first three to six days, rising to peak at 11 to 13 days before returning to prechallenge levels by about 15 days (Fig. 3.2).

The responses of lymphocytes from nine ewes, collected before and after S/C and I/V infection, to a combination of Con A and virus were compared with those to Con A alone (Table 3.5). This was done to determine whether there was any interference in the response to Con A. Nine animals were examined using efferent lymphocytes from two of these, and peripheral blood lymphocytes from all nine. Inconsistent responses were observed when both activators were added to the efferent lymphocyte cultures, but there did not appear to be any interference in the responses of cells collected before or after inoculation of the ewe (Table 3.5.1,A).

Combined stimulation of peripheral blood lymphocytes from all nine ewes (Tables 3.5.1,B and 3.5.2) also failed to induce any consistent alteration of the *in vitro* response to Con A, either before or after inoculation of the ewes with Akabane virus. That is, there was no evidence that Akabane virus causes suppression of *in vitro* lymphocyte reactivity to Con A.

3.4 DISCUSSION

It is assumed, when considering cellular responses as indicated by peripheral lymphatic duct output, that as all other efferents were tied off during cannulation the lymph collected represented the entire production of that node, and that no alternative lymphatic vessels had developed during the time of collection. It is also assumed that the *in vitro* cell reactivity measured by the lymphocyte transformation assay reflects the reactivity of lymphocytes *in vivo*, although the cautionary remarks made in Chapter Two about interpretation of mixed lymphocyte responses also apply to lymphocyte transformation responses.

Local, but not systemic,challenge with viable virus resulted in a substantial alteration in local efferent lymph characteristics. The changes were an increase in the cellular output accompanied by an increase in the frequency of blast cells in the population and a mild increase in the lymph flow rate. The initial decrease in total cell output following local challenge was rapid in onset and short in duration. This is likely to be the typical pattern observed after antigenic challenge and described by Trnka and Cahill (1980), who

suggested it may be due to a delay in the exit from the lymph node of lymphocytes which had entered the node before its antigenic challenge.

The major finding of this chapter is that while lymphocytes from control animals did not respond *in vitro* to Akabane antigen, lymphocytes from infected animals did. Peripheral blood lymphocytes and efferent lymphocytes from systemically-infected adults responded consistently when assayed several days after infection, while lymphocytes from locally-infected adults responded less consistently. There was also a difference in response according to whether or not the virus used *in vitro* had been inactivated. Lymphocytes from previously challenged animals responded to viable virus but not to inactivated virus.

It was concluded from these preliminary observations that previous exposure of an animal to Akabane virus was required if its lymphocytes were to manifest a significant response when cultivated with the virus. The *in vitro* response to viral antigen of lymphocytes from infected ewes was affected by the interval after inoculation of the donor at which the lymphocytes were collected for assay, presumably because sensitization required a finite time. The pattern of cell reactivity implies that approximately six to nine days are required for optimal *in vivo* priming of the lymphoid system. The cellular response to Akabane virus, requiring presensitization of the individual, fulfills the requirement of a specific immune response, and the virus does not appear to be mitogenic for unsensitized cells. It is possible that the Vero cells accompanying the virus particles might have been

acting as an adjuvant. If this were so, they would result in the viral challenge producing a greater cellular immune response than would occur in natural infection where Vero antigens would not be present in the inoculum. There is a further possibility concerning the accompanying Vero antigens. The changes in _in vitro_ responsiveness of lymphocytes observed after challenge of ewes may have been due to an effect of these Vero cell antigens in the inoculum, rather than to Akabane viral antigen. However, this is unlikely to account for all of the increase in the specific response to Akabane virus, as the stimulation indices plotted in Fig. 3.4 show an increased response to virus over and above that to Vero cells.

These results have implications for naturally occurring Akabane disease. The lymphocytes from unprimed animals failed to respond to Akabane virus and, even after inoculation of the ewe, the priming of the lymphocytes required six to nine days. Comparison of this time-lag with the time required for specific neutralizing antibody to appear (three to five days) implies that the effective primary immunological response to this virus is a humoral one. Cellular responses may be irrelevant in this situation.

Intravenous challenge appeared to be the most effective way of priming an animal's lymphocytes to respond to subsequent exposure _in vitro_. As regards the interpretation of the increase in cellular traffic in lymphocytes through sites of injection of virus, it is doubtful whether the cells in transition possess increased specific anti-virus reactivity. It seems at least equally possible that the major part of the

increase in cellular traffic was a response to Vero cells. It is also possible that there may be little change in the local traffic of cells through the node in the case of natural infection, particularly if the virus moves directly into blood vessels. The response of local lymph nodes, while interesting because of the indication of specific immunocompetence it gives, may not be a major component of the protective immune response to natural infection.

In contrast to the irregularity of the changes produced in the cellular reactivity of efferent lymphocytes after local challenge with virus, there was a regular increase in antibody titre in lymph from the three locally challenged sheep. Once again, the level of response, in this instance humoral, in popliteal efferent lymph rose similarly in a ewe challenged intravenously. It is relevant to mention here that adult sheep exposed to virus intravenously in another experiment (see Table 5.4) had almost all developed detectable levels of serum neutralizing antibody in serum seven days later. Examination of non-specific responsiveness of lymphocytes collected three and six days after challenge revealed a consistent fall in the proliferation induced by Concanavalin A. Taken together, these results seem to imply that the humoral response to subcutaneous challenge of adult sheep with Akabane virus is more regular and substantial than any cellular immune response that occurs.

The fourth aspect of the observed *in vitro* response to the virus that required comment was the failure of lymphocytes to respond *in vitro* to inactivated viral antigen.

Griffin and Johnson (1973), studying the response of mouse cells to Sindbis virus, also found that better stimulation was obtained with live virus antigen than with inactivated virus antigen, in contrast to the finding of a study on Herpes simplex infection of rabbits (Rosenberg, Farber and Notkins, 1972), in which inactivated antigen produced the better stimulation. Griffen et al. (1973) suggested that this difference may occur because Sindbis virus, unlike Herpes simplex, does not replicate in mononuclear cells and so does not depress DNA synthesis in immune cells. There are no reports of the ability of Akabane virus to replicate in the ovine mononuclear cells. However, data to be presented in Chapter Seven supports the possibility that replication in ovine foetal lymphocytes may occur both in vivo and in vitro. It may be that the explanation relates simply to the dose of antigen to which the animal has been exposed. A replicating virus would be expected to provide a higher total dose of antigen than the inactivated virus, and thus result in a greater stimulation of immune systems.

The response to the polyclonal activator Con A, while non-specific, remains a useful indicator of biological and immune competence of lymphocytes because the changes induced are similar to those which follow antigen-induced immune reactions (Schechter, Handzel, Attman, Nir and Levin, 1977). Peripheral blood lymphocytes and efferent lymphocytes from all of the control and infected adults responded in vitro to Con A. Although this response varied between individuals and with time after infection, the peak responses of infected ewes usually exceeded the responses of the control ewes. There is

thus no evidence that Akabane virus *in vivo* causes prolonged cellular immune suppression. There was, however, a brief drop in the *in vitro* response to Con A on the second day after inoculation, which may have been a transient virus-induced suppression. There was also evidence of a transient increase in the response of infected ewes.

The pattern of response to Con A of efferent lymphocytes from systemically and locally infected ewes was similar. These responses of efferent lymphocytes differed, however, from the observed responses of peripheral blood lymphocytes collected from systemically infected ewes. The efferent lymphocyte response showed initial depression followed by a rise in thymidine incorporation by lymphocytes collected from five days after infection. This rise reached a maximum in lymphocytes collected at about 13 days. In contrast, the initially depressed response of peripheral blood lymphocytes rose sharply to peak in lymphocytes collected at three days before immediately declining. When the capacity of Akabane virus to suppress *in vitro* lymphocyte transformation was investigated, no consistent effect of the virus on the reactivity of cells from either control or Akabane-infected ewes was found. There is thus no indication that Akabane virus has any *in vitro* suppressive or enhancing effect.

In conclusion, there was no evidence that Akabane infection of the adult sheep resulted in clinical disease or microscopical abnormalities. It did induce a specific humoral response irrespective of the route of infection. Local but not I/V infection evoked substantial change in the characteristics of the efferent lymph and its lymphocytes. There was

no evidence that Akabane virus caused *in vivo* suppression of the humoral or cellular immune systems, or suppression of non-specific cellular immune function *in vitro*. On the contrary, it appears that *in vivo* infection improved non-specific cellular immune capacity. Adult lymphocytes responded specifically to viable virus antigen *in vitro* only after *in vivo* infection. This priming was maximal after six to nine days and occurred consistently after systemic inoculation. It occurred inconsistently within the peripheral lymph node. Viable Akabane virus antigen was a more effective *in vitro* activator of lymphocytes than was the inactivated antigen.

CHAPTER 4

THE RESPONSE OF THE NEONATAL LAMB TO AKABANE VIRUS

CHAPTER 4 - THE RESPONSE OF THE NEONATAL LAMB TO AKABANE VIRUS

4.1 INTRODUCTION

As the primary interests of this investigation lay with the immune response to Akabane virus of the developing foetus and the way in which this differed from the adult response, observation of the newborn lamb as an intermediate stage of maturation was desirable. Knowledge of any effects of virus on the newborn lamb would be valuable in understanding both viral pathogenesis and the development of the lamb's immune capacity. To simplify interpretation of observations, the lambs were deprived of colostral derived maternal immunoglobulin, and were infected on the day of birth when their immune systems had had minimal exposure to, and therefore modulation by, antigenic challenge. Observations were directed to the course of any clinical disease, histopathological changes, and virus localization and persistence. Aspects of neonatal immune capacity examined included local and systemic humoral and cellular responses.

4.2 EXPERIMENTAL DESIGN

A group of four newborn lambs were challenged. Three of these were inoculated on the day of birth with 4.0×10^6 PFU of Akabane virus. One of these was by I/V injection and the remaining two by S/C injection in the area drained by the prescapular lymph node, the efferent lymphatic of which had been cannulated. The fourth lamb received a control inoculation of Vero cells in the drainage area of the prescapular lymph node. Lymph flow rate, cell count and cell type were

determined for the period that each cannula remained in place (four to nine days). Samples of a number of tissues were taken at autopsy. Spleen and thymus were selected as representatives of the lymphorecticular system, and liver and kidney as organs potentially susceptible to damage because of their high metabolic activity and detoxifying function. Thyroid and adrenal are glands of considerable importance in the young animal, and infection or lesions of these tissues might result in widespread metabolic and functional abnormalities. Since such abnormalities were unlikely to become clinically apparent in the two weeks of this experiment, during which the lambs were held in a relatively stress-free environment, these tissues were examined for indication of subclinical abnormality. Brain, spinal cord, skeletal muscle and blood were sampled because they have been previously reported as sites of Akabane virus localization or histological lesions. Samples were examined for histological abnormalities, virus and antibody titration, and fluorescent staining of viral antigen. The lambs were monitored for signs of clinical disease using parameters such as 12 hourly rectal temperature, appetite, demeanour and physical abnormalities.

Lambs were colostrum-deprived, and bottle-fed four times daily on pasteurized cows milk, diluted 1:3 for the first three days and supplemented with vitamins A, B, C and D (Pentavite, Nicholas Pty. Ltd., Chadstone, Australia).

4.3 RESULTS

In none of the lambs was there any sign of clinical disease, or of gross or microscopical lesions. Specific

neutralizing antibody was present in all three infected lambs, appearing in the sera of two of them by seven days after infection, and in sera of the third several days later (Table 4.1). The titres were similar to those in adult sera (Fig. 3.1). All tissues of all the lambs proved negative for both viable virus (by plaque assay) and for viral antigen (by immunofluorescent staining).

Of the three lambs with peripheral lymphatic cannulae and local virus or control challenge, one (36, Table 4.3, Fig. 4.2) destroyed its cannula before a complete result could be obtained. The two infected lambs (Tables 4.3 and 4.4, Figs. 4.2 and 4.3) showed a temporary drop in total cell output in the lymph collected from the challenged node between 36 and 48 hours after viral challenge but no overall rise in cell output. No alteration was observed in the rate of lymph flow or the percentage of blast cells in the efferent lymph. The control lamb (Table 4.2, Fig. 4.1) showed no alteration in any of the characteristics of the efferent lymph. Thus while the three adult ewes challenged locally with viable virus (Table 3.1) responded with increased blast cell output from the node within three days of challenge, the two similarly challenged newborn lambs had shown no such response by 3.5 days and ten days after challenge.

That is, while the affected newborn lambs mounted a specific humoral immune response resembling that of the adult, regardless of the route of infection, there was no evidence for a local cellular response, viral persistence or disease.

TABLE 4.1 - Specific neutralizing antibody levels in newborn lambs

Diameter of zone inhibition (mm)

Time(d) post inoculation	Lamb 31		Lamb 61		Lamb 36		Lamb 8
	lymph	serum	lymph	serum	lymph	serum	serum
1	0		0		0		
2	0		0				
3	0				0		
4	0		0		0		
5	0		0				
6			0				
7		0	0			13	7
8			0	0			
9			0				
10		0				12	17
12				11			
14		0				13	12

Lamb 31 received Vero control, the remainder were challenged with viable Akabane virus.

TABLE 4.2 - Efferent lymph data from lamb # 31 after local challenge with Vero antigen.

SAMPLE	TIME TOTAL	FLOW ML/HR	TOTAL CELLS /ML	TOTAL CELLS /HR	LARGE CELLS /ML	LARGE CELLS /HR	%
1	6.25	4.7	2.70	12.79	0.033	0.160	1.25
2	14.83	3.1	12.21	38.55	0.245	0.776	2.01
3	27.50	4.1	22.90	95.65	0.483	2.020	2.11
4	38.58	2.9	26.71	78.34	0.656	1.925	2.45
5	50.83	3.2	13.74	44.08	0.218	0.700	1.58
6	62.66	2.2	13.38	30.09	0.340	0.765	2.54
7	75.25	2.9	11.52	33.42	0.412	1.195	3.57
8	86.83	2.3	12.13	27.97	0.288	0.664	2.37
9	99.83	3.7	10.54	39.09	0.279	1.036	2.65
10	110.83	2.7	13.15	36.00	0.423	1.158	3.21
11	123.83	3.7	13.13	49.59	0.440	1.664	3.35
12	134.49	2.0	12.09	24.27	0.482	0.968	3.99
13	145.99	0.2	7.85	1.70	0.390	0.084	4.97

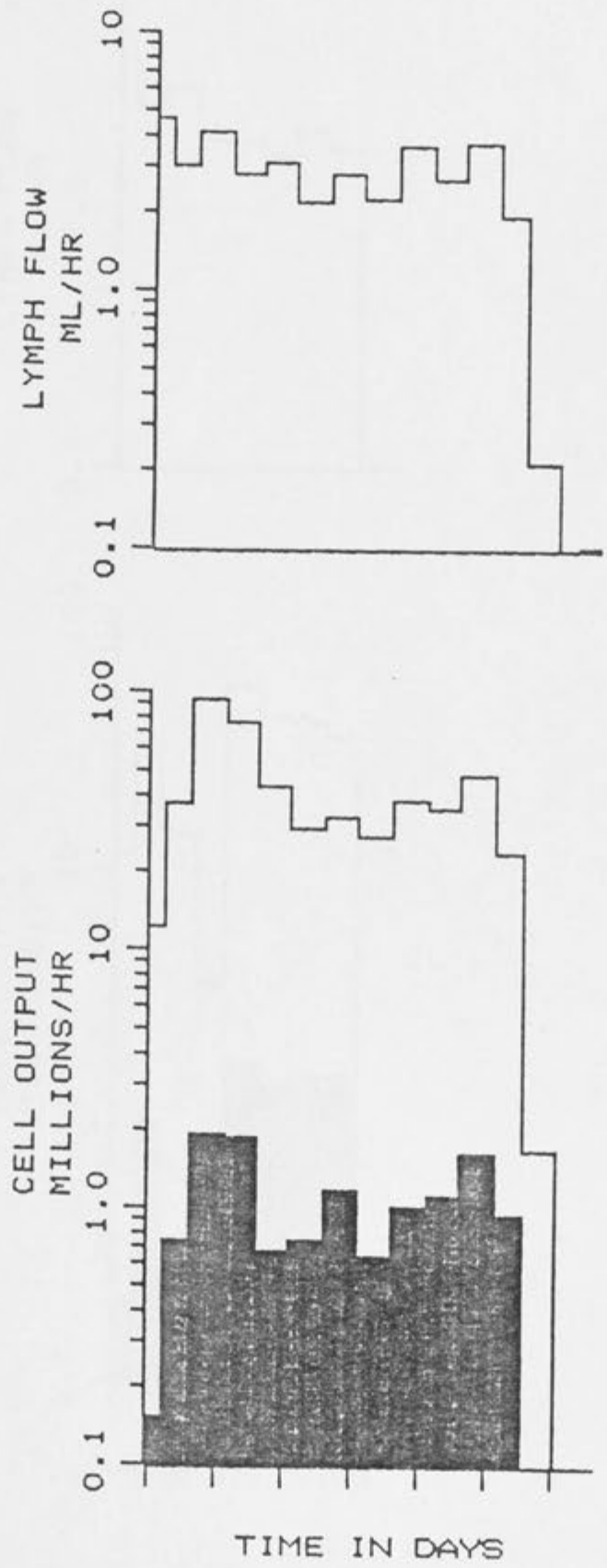
cells x 10^6

TABLE 4.3 - Efferent lymph data from lamb #36 after local challenge with viable Akabane virus.

SAMPLE	TIME TOTAL	FLOW ML/HR	TOTAL CELLS /ML	TOTAL CELLS /HR	LARGE CELLS /ML	LARGE CELLS /HR	%
1	5.75	3.9	3.03	11.87	0.057	0.226	1.90
2	14.50	2.5	12.34	31.75	0.416	1.072	3.37
3	27.25	3.0	4.60	14.15	0.063	0.193	1.36
4	38.33	1.8	1.97	3.71	0.079	0.150	4.04
5	50.50	1.8	28.49	52.22	1.003	1.838	3.52
6	62.33	1.6	24.58	40.09	0.905	1.476	3.68
7	74.91	2.1	17.99	37.88	0.788	1.661	4.38
8	86.58	1.7	19.32	33.95	0.926	1.628	4.79

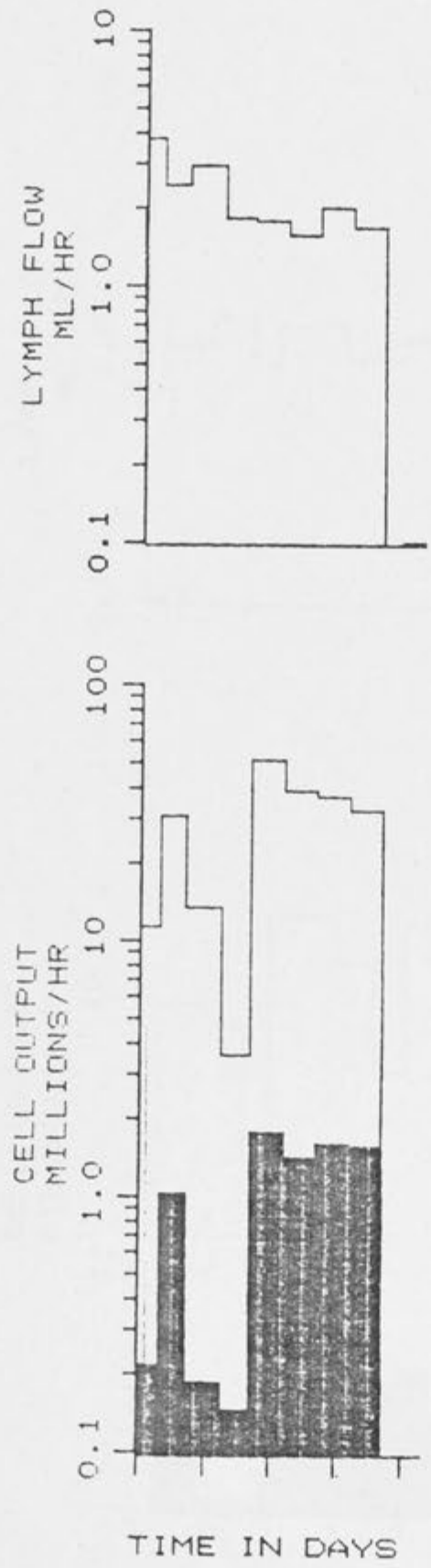
cells x 10^6

FIGURE 4.1 - Efferent lymph data from lamb # 31
after local challenge with Vero antigen.



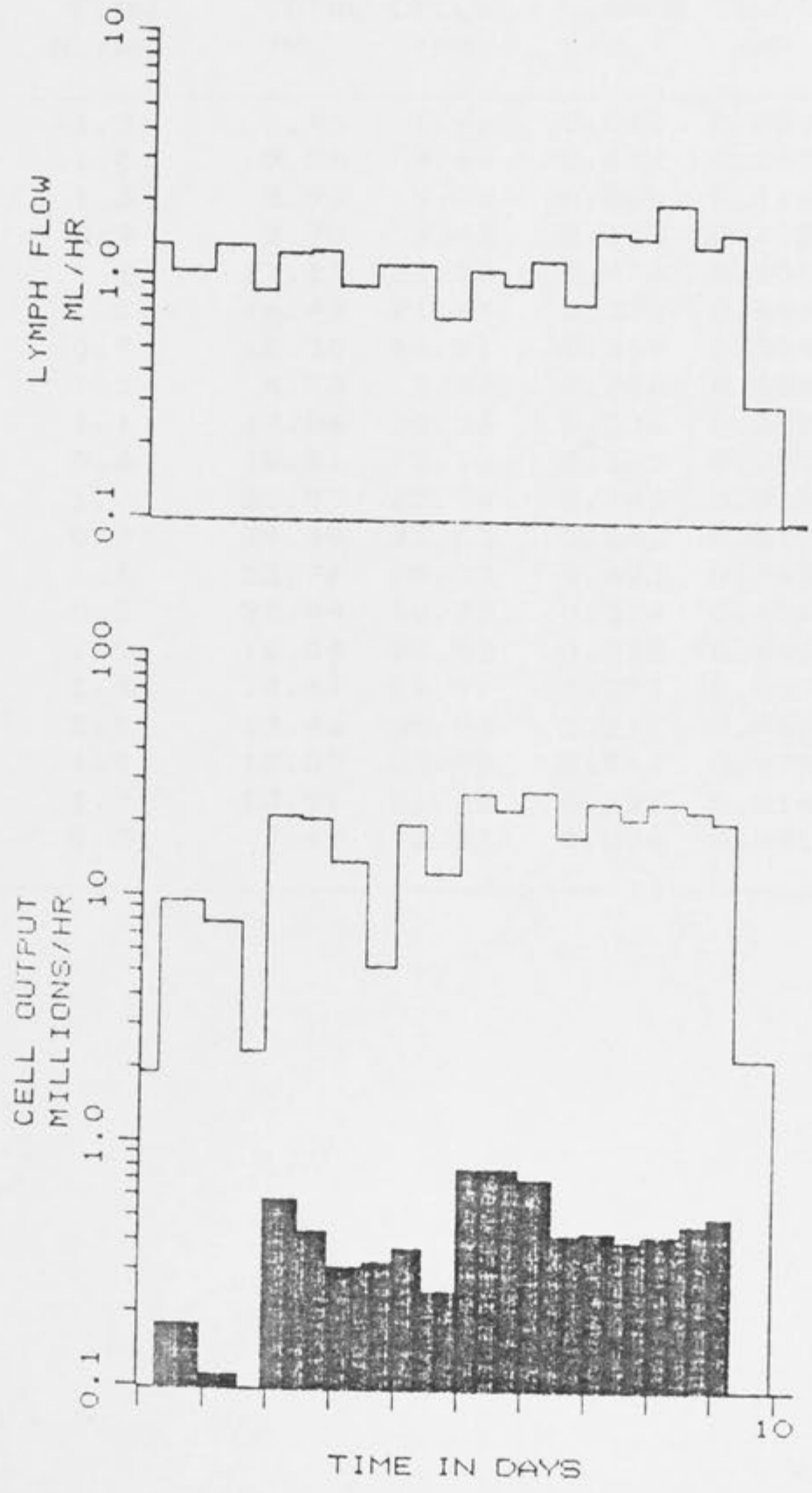
The lamb was locally challenged at day 0.
The hatched area represents the output of blast cells,
while the open histogram represents total cell output.

FIGURE 4.2 - Efferent lymph data from lamb # 36 after local challenge with viable Akabane virus.



The lamb was locally challenged at day 0.
The hatched area represents the output of blast cells, while the open histogram represents total cell output.

FIGURE 4.3 - Efferent lymph data from lamb # 61
after local challenge with viable Akabane virus.



The lamb was locally challenged at day 0.
The hatched area represents the output of blast cells,
while the open histogram represents total cell output.

TABLE 4.4 - Efferent lymph data from lamb # 61 after local challenge with viable Akabane virus.

SAMPLE	TIME TOTAL	FLOW ML/HR	TOTAL CELLS /ML	TOTAL CELLS /HR	LARGE CELLS /ML	LARGE CELLS /HR	%
1	7.08	1.3	1.43	1.96	0.042	0.058	2.97
2	22.75	1.0	9.29	9.89	0.171	0.182	1.84
3	37.75	1.3	5.90	8.02	0.084	0.114	1.42
4	46.75	0.9	2.70	2.43	0.113	0.102	4.19
5	59.75	1.2	17.17	21.80	0.473	0.601	2.75
6	71.41	1.3	16.49	21.45	0.338	0.440	2.05
7	85.41	0.9	15.30	14.21	0.349	0.324	2.28
8	95.66	1.1	4.73	5.48	0.288	0.334	6.10
9	106.91	1.1	17.86	20.28	0.336	0.382	1.88
10	120.16	0.6	18.81	13.16	0.365	0.255	1.94
11	133.16	1.0	25.53	27.79	0.746	0.812	2.92
12	142.99	0.9	24.48	23.53	0.843	0.810	3.44
13	156.16	1.1	23.76	28.33	0.623	0.743	2.62
14	167.33	0.8	22.44	18.32	0.534	0.436	2.37
15	180.16	1.5	16.04	25.52	0.275	0.438	1.71
16	190.49	1.4	14.67	21.97	0.271	0.407	1.85
17	205.41	2.0	12.46	25.83	0.211	0.437	1.69
18	215.16	1.4	17.07	23.95	0.341	0.478	1.99
19	223.58	1.5	13.51	21.25	0.327	0.514	2.42
20	239.16	0.3	7.47	2.31	0.294	0.091	3.94

cells x 10^6

4.4 DISCUSSION

The technique of peripheral lymphatic cannulation in the neonatal lamb encountered problems not found with the technique in either adults or foetuses. The young lamb is very active, and even when the cannula was anchored at the skin and protected by stocking, in most lambs the cannulae worked out of the lymph vessels within several days of surgery. It was also necessary to separate cannulated lambs from their mothers to avoid displacement of the cannula by maternal licking.

Neither the subcutaneous nor the intravenous route of infection employed in this experiment resulted in disease in these neonatal lambs. In retrospect, use of the intracerebral route could have been valuable to distinguish between failure of pathogenicity due to a target tissue insusceptibility and that resulting from curtailment of viral access to the target tissue as a consequence of an immune response or maturation of the blood/brain barrier. It is likely that mature central nervous tissue may remain susceptible to the virus despite the lack of clinical disease following inoculation by the normal route, as two other endemic arboviruses - Kunjin (Spradbrow and Clark, 1966) and Australian encephalitis (Miles, 1952) - have been shown to cause overt disease in ruminants only after inoculation into the brain.

It is evident from the appearance of specific neutralizing antibody that infection occurs in the newborn lamb, which can then mount a specific immunological response to the virus. The concurrent failure of lesions to develop and of virus to persist implies two possibilities. Either the lamb was no

longer susceptible to virus-induced damage, or it had developed a sufficiently effective immunological capacity to inactivate or eliminate the virus before it could produce abnormalities. The neonatal lamb developed levels of specific antibody similar to those of adults, although the time of first appearance was delayed for two to three days later than in the adult. Since these lambs had no access to colostrum and therefore no maternal antibody, it is inferred that the neonate is capable of mounting a specific humoral response to Akabane virus that resembles that of the immunologically competent adult.

However, local challenge of a peripheral lymph node with viable Akabane virus failed to alter the output from the efferent lymphatic vessel. Since the in vitro reactivity of the neonatal lymphocytes was not assayed, it is not possible to say whether a systemic cellular immune response was induced by the virus. It might be that the neonatal lamb does not mount such a response, perhaps because of immaturity. Comparison of the absence of detectable local cellular response to the virus in the neonate with the consistent cellular response to local infection of the adult raises a further question. It is possible that the adult response observed was in fact a secondary rather than a primary one. Since all ewes in the flock were seronegative with respect to antibody against Akabane virus, this would imply that these three ewes had been previously exposed, and that their antibody levels had since dropped below detectable limits. In addition, given the mode of transmission of Akabane virus, and the fact that the experimental animals were bred and maintained together, it is unlikely that only a small proportion of the flock would be

infected. It therefore seems more likely that the maternal response was primary.

In conclusion, while both local and systemic challenge of the colostrum-deprived newborn lamb with Akabane virus stimulated a specific immune response of adult type, there was no evidence of clinical disease, viral persistence or any local cellular response.

CHAPTER 5

TRANSPLACENTAL AKABANE VIRAL INFECTION OF THE SHEEP FOETUS

CHAPTER 5 - TRANSPLACENTAL AKABANE VIRAL INFECTION OF THE SHEEP FOETUS

5.1 INTRODUCTION

The experiments described in Chapters Five to Seven were intended to trace the course of Akabane infection in foetal lambs in relation to the development of humoral and cell-mediated immunological reactivity. Akabane virus is of considerable immunological interest because of its capacity to cross the placenta and enter the foetal lamb substantially before any capacity to mount an immune response becomes demonstrable (Parsonson et al, 1981a). Apart from the opportunity that this offers to test for induction of immunological tolerance of viral antigens, the fact that there is a variation in pathological effects consequent upon viral exposure at different foetal ages (Narita et al, 1979) provides an opportunity to examine the extent to which varying degrees of immunological maturation can influence the nature of the pathological lesions produced.

The pathological consequences of exposure of an animal of any age to Akabane virus will reflect the interaction between direct effects of the virus on host tissues and the response, immunological and otherwise, of the host to viral invasion. Additional factors influencing the outcome will be the stage of maturation attained by target organs by the time of infection and any modulating influence of the placenta on intensity or duration of foetal exposure to virus. This study, therefore, aimed to obtain information on the effects of foetal age, morphological development, placental development and

immune response on the course of the infection.

In order to understand the pathogenesis of a viral infection and the relative contributions of virus and host to the resulting disease, it is essential to describe the fate of that virus within the host. Such aspects as its capacity to infect, survive and replicate in different tissue and cell types, while subject to influence by host responses, permit detection of any correlations between localization of virus and the occurrence of pathological changes. Knowledge of such associations is fundamental to any interpretation of the mechanisms by which viral pathology is produced.

This and the two succeeding chapters describe some aspects of Akabane virus behaviour in foetuses infected at a range of ages (31 to 126 days). Both transplacental and direct routes of infection were used. Foetuses were infected over a range of ages, from ages substantially earlier than those suggested for the acquisition of immunological competence (Parsonson et al, 1981a; Miyasaka and McCullagh, 1981) when induction of tolerance would seem likely, to late gestation, when the foetus is capable of a substantial, albeit incomplete, range of immunological responses.

The normal route of infection of the foetus is transplacental, and the group of foetuses described in this chapter was exposed to the virus only by transplacental transmission from the inoculated dam. The virus has been shown to cause foetal deformities in sheep only when the mothers are infected between 29 and 81 days, even though there is evidence of transplacental transmission of the virus up to 101 days

(Narita et al, 1979; Hashuguchi et al, 1979). While these ages appear to vary with the isolate used, other isolates also exhibited a progressive diminution of effect as foetal age at time of maternal infection increased. This diminution was shown by the fact that failure of virus injection into the ewe to evoke pathological consequences preceded failure to cross the placenta by 14 days (Parsonson et al, 1977). Attempted infection outside the 29 - 101 day range has not been reported, nor has infection with the CSIRO 16 strain used in the present experiments been attempted outside 32 - 36 days. Thus, Akabane virus appears to retain the capacity to cross the placenta for at least the second and third months of pregnancy, although a teratogenic capacity has not been reported if infection occurred in the second half of pregnancy.

This chapter describes the examination of foetuses aged between 22 and 75 days, after infection of their mothers at 18 days or 31 to 44 days of gestation. A single time point measurement of response was made when the foetus was recovered. To elucidate further the role of the placenta in foetal disease, an attempt was made to determine whether the virus would cross to the foetus before implantation (i.e. before 26 days). Electron microscopy was used to search for virions in the foetal tissues. Chapters Six and Seven describe experiments in which the results of direct viral inoculation of foetuses were examined.

5.2 EXPERIMENTAL DESIGN

Disease can be followed in a post-parturient subject by serial monitoring of many parameters, both morphological, for

example bloodcounts, and functional, as in hormonal sufficiency tests, in addition to clinical observations. Such monitoring is rarely possible in foetuses except in late gestation, where the use of indwelling cannulae permits observation of changes in blood, lymph and foetal fluids. Thus study of disease in foetuses is usually limited to single time-point observations made at the time of foetal recovery and autopsy. In the following study and in that to be described in Chapter Six, the younger foetuses were examined only once for signs of disease and immunocompetence, while the older ones, to be described in Chapter Seven, were also submitted to serial observations of some parameters.

5.2.1 Five ml of medium containing 5.0×10^7 PFU of viable Akabane virus were injected into the middle uterine artery supplying the pregnant horn of each of three ewes at day 18 of pregnancy. Four days later, the embryos were recovered and fixed for electron microscopy as described in Materials and Methods. Sections of central nervous tissue and mesenchyme (200 cells of each) were examined for the presence of Akabane virions.

5.2.2 One ml of medium containing 10^7 PFU of viable virus was injected via the jugular vein into another 28 pregnant ewes between 31 and 44 days gestation. The 32 foetuses produced by these ewes were removed for examination at intervals between 37 and 75 days gestation.

Fourteen of these foetuses between 38 and 70 days gestation had 50 μCi methyl ^{3}H-thymidine injected into the umbilical vein 30 minutes before recovery, and autoradiographs

were prepared from the tissues taken at autopsy as described in Materials and Methods.

Twenty-nine of the foetuses were examined for virus distribution and titre, as described in Materials and Methods. Viral titre was measured in allantoic and amniotic fluids, blood, brain, spinal cord, skeletal muscle (biceps femoris and quadriceps groups), liver, spleen, thymus, interplacentomal foetal membranes (chorioallantoic and amniotic), placentome and, in some instances, in lung and lymph nodes. Some of these tissues (brain, skeletal muscle, liver,spleen, thymus, foetal membrane, placentome and sometimes spinal cord) were also frozen in liquid nitrogen for immunofluorescent staining for viral antigen as described in Materials and Methods. Not all of these tissues were collected from the younger foetuses, either because they were inadequately differentiated or because there was insufficient tissue to assay.

Thirty of the foetuses were examined for gross and microscopic lesions.

Liver cells from 13 foetuses were assayed for in vitro transformation response to Con A and to live Akabane virus. Cells from some of these foetuses were also examined for responsiveness to PHA, LPS and PWM.

Sera or allantoic fluids from 17 foetuses were assayed for the presence of specific neutralizing antibody.

Twenty of the ewes were bled at weekly intervals and their sera assayed for specific neutralizing antibody against Akabane virus.

5.2.3 An additional 14 ewes which had not been inoculated with virus, and their 15 foetuses, were used as controls for the treatments outlined in 5.2.2.

Ewes carrying control foetuses were not inoculated with non-infected suckling mouse brain. Given the clear cut nature of the anticipated foetal lesion, their inoculation was not regarded as necessary.

Foetal tissues had a tendency to autofluoresce. For this reason each stained section was compared with its control, and only fluorescence that could be confirmed as specific in this way was recorded as positive. Therefore the figures for presence of viral antigen may be underestimates. As a further check, the examination of equivocal specimens was repeated and these were counter-stained with Evan's blue to suppress non-specific fluorescence.

Cell types were difficult to distinguish in cryosection, largely because they were not stained for tissue differentiation. Therefore fine distinction between cell types was not attempted. This problem could be overcome by working with duplicate paraffin sections: staining one with haematoxylin/eosin, and the other with fluorescein, so that the fluorescing cells could then be identified by comparison.

5.3 RESULTS

Pre-implantation embryos

All four embryos from ewes inoculated with Akabane virus at 18 days gestation were alive and grossly normal when recovered four days later. Examination of the central nervous system tissues or of the undifferentiated mesenchyme under

the electron microscope did not detect virions, despite a search of similar extent to that described in Chapter Seven which detected Akabane virions in foetal lymphocytes (that is, examination of 200 cells of each type per foetus).

Post-implantation foetuses

Thirty-two foetuses were recovered from infected ewes at 37 to 75 days gestation. In all of the 14 foetuses that had been inoculated with radio-isotope 30 minutes before recovery, autoradiographs (Plate 5.1) showed that the label had been taken up principally by the following tissues: liver - parenchymal and haemopoietic cells (chiefly the latter), skeletal muscle (chiefly myocytes, but also fibrocytes), and heart (cardiac muscle cells, especially in sub-endocardial regions). No labelled cells were observed in brain.

The distribution of viable Akabane virus is shown in Table 5.1, of viral antigen in Table 5.2 and of morphological abnormalities in Table 5.3. Typical histological lesions are illustrated in Plates 6.2 and 6.6. The youngest foetus recovered (#24 at 37 days) had had a maximum of six days' exposure to the virus, and probably slightly less because of the time taken for the virus to cross the placenta. Neither viable virus nor viral antigen were detected and the foetus was morphologically normal on gross and light microscopical examination. In foetus #30, recovered at 38 days after a maximum of seven days' exposure, viable virus was detected only in placentome and chorioallantoic membrane, while neither viral antigen nor morphological abnormality was detected. Foetus #22 was recovered at 40 days after a maximum of nine days'

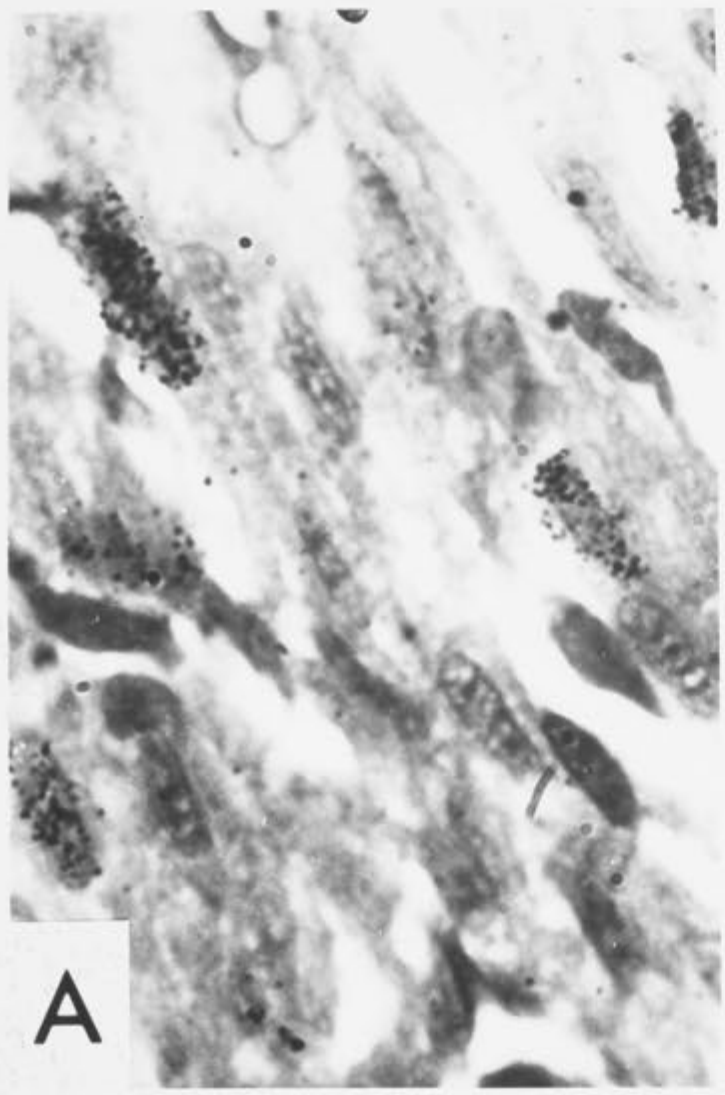
PLATE 5.1 Autoradiographs of methyl ^{3}H-thymidine incorporation by cells of foetuses infected with Akabane virus.

A: # 51, aged 50d, left cardiac ventricle. The radioisotope has been incorporated by many of the myocytes.
Azure - A, x 1000.

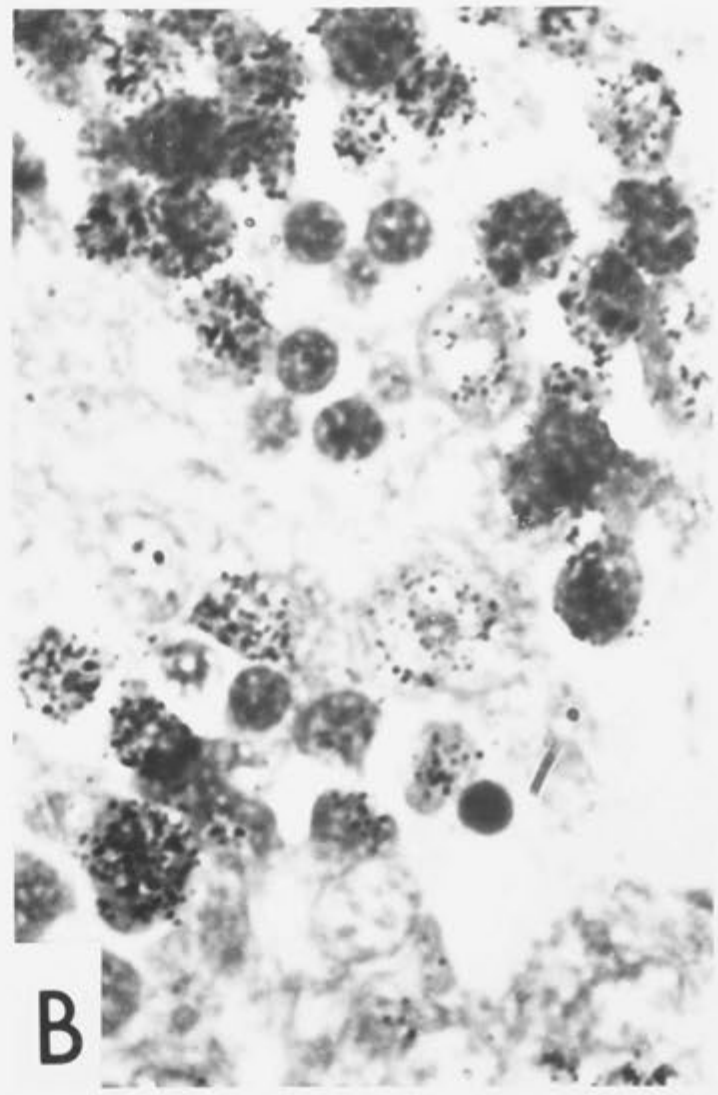
B: # 51, aged 50d, liver. The radioisotope has been incorporated by most of the haemopoietic cells and precursors, and by some of the parenchymal hepatocytes.
Azure - A, x 1000.

C: # 51, aged 50d, skeletal muscle (biceps femoris). Myocytes and mesenchymal cells have incorporated the radioisotope.
Azure - A, x 1000.

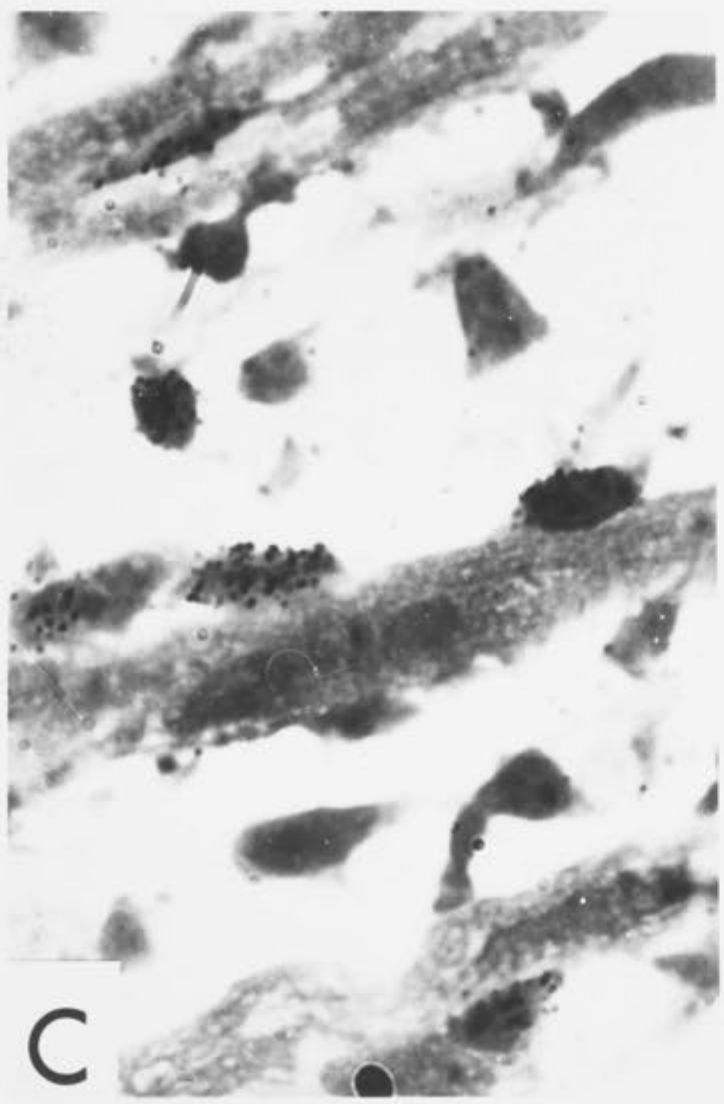
D: # 36, aged 73d, skeletal muscle (biceps femoris). The radioisotope has been incorporated by the myocytes.
Azure - A, x 1000.



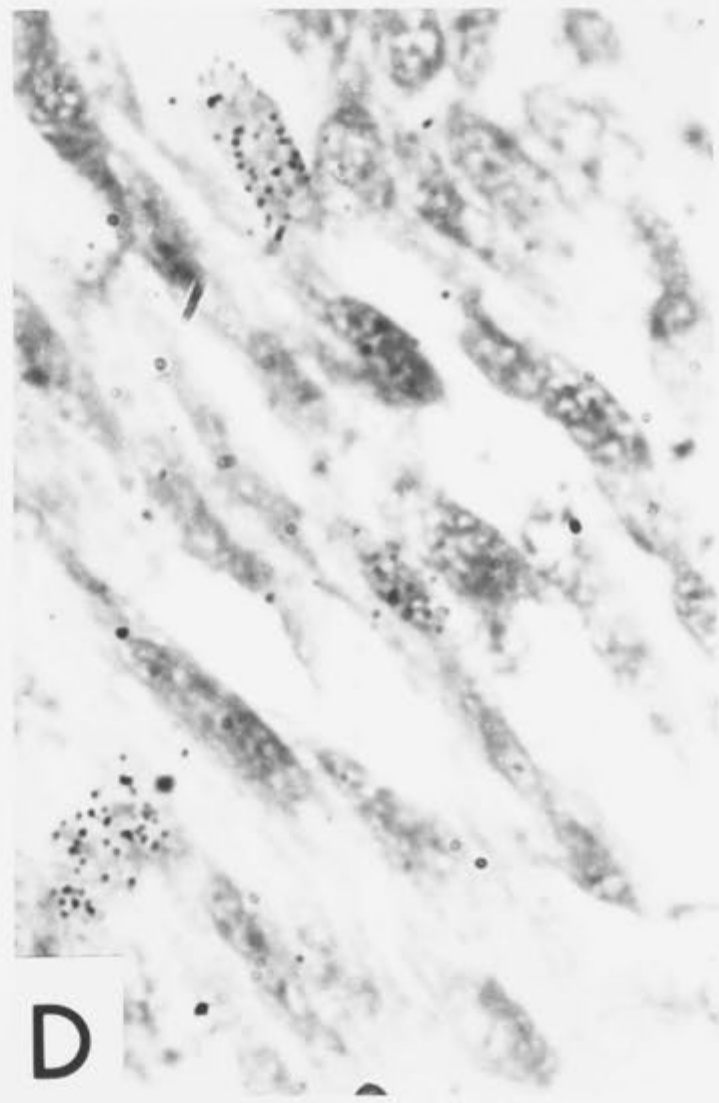
A



B



C



D

TABLE 5.1.1 - Distribution of viable Akabane virus in ovine foetuses following transplacental infection

Sheep	24	30	22	29	21	25	55	41	23	51	39	45	43	S32	32	28
Age (d) at inoculation	31	31	31	31	31	33	31	31	32	34	31	32	32	31	34	33
Age (d) at recovery	37	38	40	41	44	45	46	47	49	50	52	52	55	55	56	56
Cerebrum	-	-	12	-		25		-	+	20	-	10	-	7	+	15
Placentome		5	+	5				$5.0x10^2$	-	+	-	+	$2.5x10^3$	+	$1.5x10^2$	+
Foetal membrane		5	5	-	-	5	$2.5x10^2$	+		+	-	-	-	+	-	+
Skeletal muscle	-	-	5	-	-	-	$9.5x10^2$			-	5	-	-	20	10	+
Liver	-	-	-	-						-	-	-		-		-
Spleen																
Thymus																-
Blood											-					-
Allantoic fluid	-		-	-		10	-	-	-	-	+	20	5	-	-	-
Amniotic fluid	-		-	-		-	-	-	-	-	+		5	10	-	-

(+) = cytopathic effect.

(-) = no cytopathic effect or titre.

Virus titre of tissues is expressed as PFU/ml of the 10% suspension (See Materials and Methods).

TABLE 5.1.2 - Distribution of viable Akabane virus in ovine foetuses following transplacental infection, continued.

Sheep	30B	26	31	53	49	40B	50	46	33	36B	37B	S46
Age (d) at inoculation	32	34	32	32	31	40	32	31	44	40	40	31
Age (d) at recovery	59	62	63	63	66	67	69	69	71	73	75	75
Cerebrum	5		+	-	+	+	+			+	+	-
Placentome	+	$2.0x10^2$		+	-	-	-	-	$6.5x10^2$	10	$2.5x10^3$	20
Foetal membrane				+	-	$1.0x10^2$	+	$1.0x10^2$	50	$1.0x10^2$	40	-
Skeletal muscle	10	+	5	5	-	-	-		-	+	10	20
Liver		-	-				-		-			-
Spleen			-	-		-		-	-	-	-	-
Thymus	-		-	-	-	-		-		-		-
Blood	-	-			-	-	-		8	+	-	-
Allantoic fluid	-		-	12	-	-		-	25		-	-
Amniotic fluid	-		-		-	-		-			-	-

(+) = cytopathic effect.

(-) = no cytopathic effect or titre.

Virus titre of tissues is expressed as PFU/ml of the 10% suspension.

TABLE 5.2.1 - Distribution of Akabane viral antigen in foetuses following transplacental infection

Sheep	24	30	22	29	21	25	55	41	23	51	39	45	43	S32	32	28	30B	26	31
Age (d) at inoculation	31	31	31	31	31	33	31	31	32	34	31	32	32	31	34	33	32	34	32
Age (d) at recovery	37	38	40	41	44	45	46	47	49	50	52	52	55	55	56	56	59	62	63
Stained antigen:																			
Cerebrum	-	-	-	+	-	+	-	+	-			+	+	+	+	+	+	+	+
Placentome	-	-	-	+	-	+		+	+	+	+	-	+	+	+	+	+	-	-
Skeletal muscle	-	-	-	+	-	+		-	-	-	+	+	+	-		-	+	+	-
Foetal membrane	-	-	-	-	-	-	+		+	-	-	+		+	+	+		+	+
Liver	-	-	-	+	-	-	-	-	-		-	+	-	-		-	-	-	
Spleen											-	-		-		-		-	-
Thymus											-	-	-	-		-	-	-	-

The antigen was detected by specific immunofluorescent staining.

+ = antigen present

- = antigen not detected.

TABLE 5.2.2- Distribution of Akabane viral antigen in foetuses following transplacental infection, continued.

Sheep	53	49	40B	50	46	33	36B	37B	S46
Age (d) at inoculation	32	31	40	32	31	44	40	40	31
Age (d) at recovery	63	66	67	69	69	71	73	75	75
Stained antigen:									
Cerebrum	+		+	+	/	+	+	+	+
Placentome	+		-	+	+	+	-	+	
Skeletal muscle	+		-	-		+	-	-	+
Foetal membrane	-		+	+	+	-	-	+	-
Liver	+		-	-	-	-	-	-	+
Spleen	-		-	-	+	-	-	-	-
Thymus	-		-	-	+	-	-	-	-

The antigen was detected by specific immunofluorescent staining.

+ = antigen present

- = antigen not detected

/ = no cerebrum present.

TABLE 5.3.1 - Gross and histological abnormalities of foetuses transplacentally infected with Akabane virus

Sheep	24	30	22	29	21	25	55	41	23	51	39	45	43	S32	32	28	30A
Age (d) at inoculation	31	31	31	31	31	33	31	31	32	34	31	32	32	31	34	33	32
Age (d) at recovery	37	38	40	41	44	45	46	47	49	50	52	52	55	55	56	56	59
Gross Pathology	-	-	-	-	-	-	-	-	-	+	+	-	-	+	-	-	-
Histopathology:																	
Cerebrum	-	-	-	+	-	-	-	-	-	+	++	+	+	+	++	+	+
Cerebellum						-											
Spinal cord		-		-										-			
Meninges	-	-	-	-		-	-	-	-	-	-	-	-			-	+
CNS infiltration	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Placentome	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Skeletal muscle	-	-	-	-	-	-		-	-	-	+	+	+	-	+	+	+

Abnormalities were scored (-) - (++++), where (++++) = total loss of the tissue.

CNS = central nervous system tissue.

Twins are referred to as A and B.

TABLE 5.3.2 - Gross and histological abnormalities of foetuses transplacentally infected with Akabane virus, continued.

Sheep	30B	26	31	53	49	40A	40B	50	46	33	36A	36B	37A	37B	S46
Age (d) at inoculation	32	34	32	32	31	40	40	32	31	44	40	40	40	40	31
Age (d) at recovery	59	62	63	63	66	67	67	69	69	71	73	73	75	75	75
Gross pathology	-	-	+	-	-	-	-	-	++++	-	+	+	+	-	-
Histopathology:															
Cerebrum	+	++	++		+++	++	++	+	++++	+++	+++	++			
Cerebellum								-	++++		-	-		-	-
Spinal cord															-
Meninges								+	++++	+++	+	+		+	-
CNS infiltration	-	-	-		-	-	-	-	/	++	-	-		-	-
Placentome	-	-	-	-	+		+	-	++	+		-		+	-
Skeletal muscle	+	+	+		++	-	+	+	++	++	+	+		+	+

Abnormalities were scored (-) - (++++), where (++++) = total loss of the tissue.

/ = unknown due to absence of central nervous tissue.

exposure, and viable virus was found in brain, placentome, chorioallantoic membrane and skeletal muscle. Neither viral antigen nor morphological abnormality was detected. Foetus #29, recovered at 41 days after a maximum of 10 days' exposure, showed viable virus only in placentome although viral antigen was detected in brain, placentome, skeletal muscle and liver. Brain was the only tissue to show pathological changes, and these were only visible microscopically. They consisted of focal necrosis, malacia and cavitation of the mid-brain. Foetus #21 had had a maximum of 13 days' exposure, and examination at 44 days failed to reveal viable virus, viral antigen or morphological abnormality. In foetus #25, recovered at 45 days after a maximum of 12 days' exposure, viable virus was detected in placentome, foetal membranes and allantoic fluid. Viral antigen was present in brain, placentome and skeletal muscle, and the foetus was morphologically normal. Foetus #55 had a maximum of 15 days' exposure, and when recovered at 46 days had detectable viable virus in foetal membranes and skeletal muscle and viral antigen in foetal membranes but no morphological abnormalities. Foetus #41 was recovered at 47 days, after a maximum of 16 days' exposure. Viable virus was detected in placentome and foetal membranes, and viral antigen in brain and placentome. This foetus was also morphologically normal. Foetus #23 was recovered at 49 days, after a maximum of 17 days' exposure. Viable virus was detected only in brain by its cytopathic effect on Vero cells, viral antigen was present in placentome and foetal membranes, but its tissues were again morphologically normal.

Foetus #51 was the first of the 50 to 70 day group to be

examined. When examined at 50 days, it had had a maximum of 16 days' exposure. Viable virus was recovered from brain, placentome and foetal membranes and viral antigen was detected in placentomes. Gross morphological changes were present and consisted of diminution in the size of the cranium and hindquarters. Histological examination revealed focal necrosis, malacia and cavitation of the intermediate layer of the cerebral hemispheres. Foetus #39 was recovered at 52 days, after a maximum of 21 days' exposure. Viable virus was detected in skeletal muscle and amniotic and allantoic fluid and viral antigen in placentome and skeletal muscle. This foetus was superficially abnormal (see Plate 5.2.b), with diminished size of hindquarters, hindlimb flexion and brachygnathism. Histopathological lesions similar to those seen in the younger foetuses were also present in the cerebral hemispheres, and in addition, focal necrosis was observed in skeletal muscle. Foetus #45 was also recovered for examination at 52 days having a maximum possible exposure time of 20 days. Viable virus was recovered from brain, placentome and allantoic fluid, and antigen was detected in brain, skeletal muscle, foetal membranes and liver. While no gross abnormalities were visible, the cerebral hemispheres and skeletal muscle contained characteristic microscopic necrotic lesions. Skeletal muscle also showed evidence of focal haemorrhage. Foetus #43, recovered at 55 days after a maximum of 23 days' exposure, had recoverable virus in placentome and allantoic and amniotic fluids. Viral antigen was detected in brain, placentome and skeletal muscle. No superficial abnormalities were seen, although characteristic necrotic lesions were

PLATE 5.2 Superficial gross abnormalities of foetuses infected with Akabane virus.

a: # 43, aged 49d, uninfected control.

b: # 39, aged 52d, infected. Superficial abnormalities consisted of underdevelopment of the hindquarters, hindlimb flexion and brachygnathism.

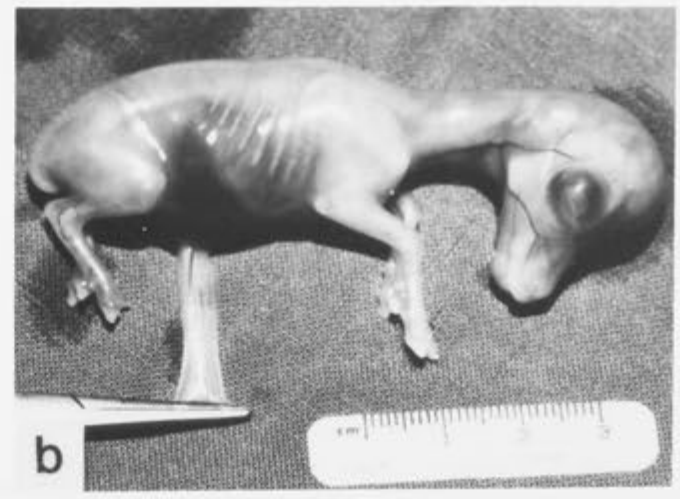
c: # 44, aged 62d, uninfected control.

d: # 31, aged 63d, infected. Superficial abnormalities consisted of underdevelopment of the hindquarters, hindlimb flexion and cutaneous haemorrhages.

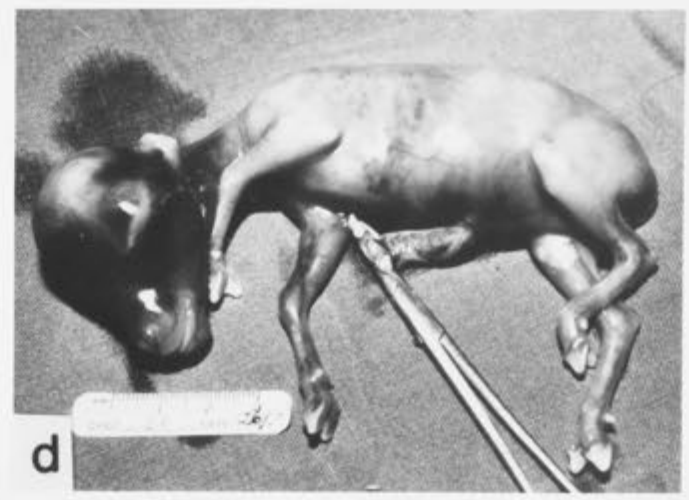
e: # 381, aged 75d, uninfected control.

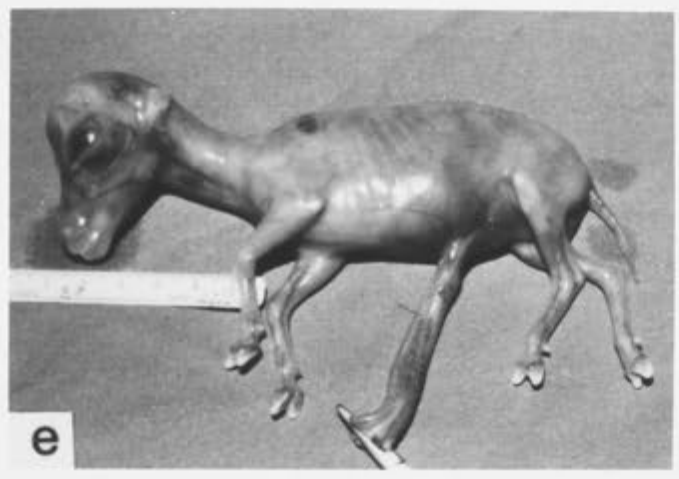
f: # 46, aged 69d, infected. Superficial abnormalities consisted of underdevelopment of the hindquarters, severe arthrogryposis and kyphosis, brachygnathism, micrencephaly, cutaneous haemorrhages, subcutaneous oedema, abdominal distention and very poor circulation.

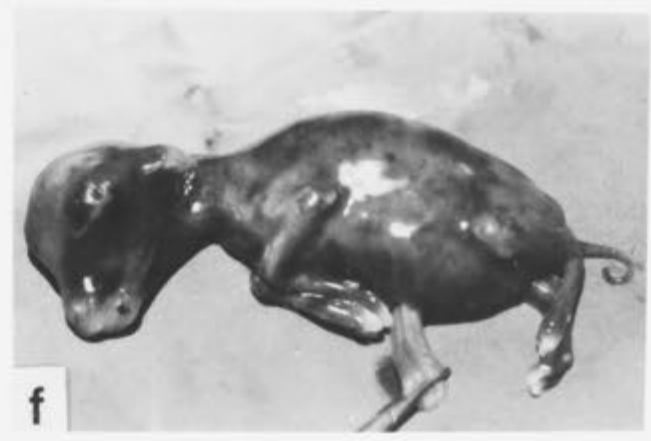

a


b


c


d


e


f

visible microscopically in cerebral hemispheres and skeletal muscle. Foetus #S32 was also examined at 55 days, after a maximum of 24 days' exposure. Viable virus was present in a wide range of tissues - brain, placentome, foetal membranes, skeletal muscle and amniotic fluid. Viral antigen was present in brain, placentome and foetal membranes. A number of gross lesions were visible, namely non-haemorrhagic oedema of the head and brisket, kyphosis, flexion of all limbs, and diminution in the size of the hindquarters. Characteristic necrotic lesions were visible microscopically only in cerebral hemispheres. Foetus #32 was recovered at 56 days after a maximum of 22 days' exposure. Viable virus was detected in brain, placentome and skeletal muscle, and antigen in brain, placentome and foetal membrane. Skeletal muscle was not examined for viral antigen. This foetus was grossly normal, despite the presence of the usual necrotic lesions in skeletal muscle, cerebral hemispheres and mid-brain. The thyroid follicles contained less colloid than normal. Foetus #22 was also recovered at 56 days, after a maximum of 23 days' exposure. Viable virus was recovered only from brain, placentome, foetal membranes and skeletal muscle, while antigen was detected only in brain, placentome and foetal membranes. Superficial morphological appearance was normal, while microscopic examination revealed the usual necrotic lesions of the cerebral hemispheres and skeletal muscle. There was some fibrous tissue replacement of skeletal muscle fibres. Ewe #30 had twins which were examined at 59 days after a maximum of 27 days' exposure. One of these (#30A) was only examined for morphological changes. This examination failed to reveal

gross abnormalities. Microscopical changes were present in the brain, with hyperaemia of meninges and parenchyma, and focal necrosis, malacia and cavitation of cerebral hemispheres and mid-brain. Focal necrosis, with a decrease in the number and diameter of fibres, had occurred in skeletal muscle. In the case of the other twin (#30B), both viable virus and viral antigen were recovered from brain, placentome and skeletal muscle. This twin was also grossly normal while containing characteristic microscopic necrotic lesions in its cerebral hemispheres and skeletal muscle. Some fibrous tissue replacement of necrotic skeletal muscle fibres had occurred.

Foetus #26 was examined at 62 days, after a maximum of 28 days' exposure. Viable virus was present in placentome and skeletal muscle, while brain was not examined for virus. Viral antigen was present in brain, skeletal muscle and foetal membranes. Gross pathological changes were not seen, but moderately severe necrotic lesions were visible microscopically in cerebral hemispheres. Cerebral meninges were hyperaemic, focal necrosis had occurred in skeletal muscle, and the quantity of thyroid colloid appeared to be subnormal. Foetus #31 was recovered at 63 days, after a maximum exposure time of 31 days. Viable virus was recovered from brain and skeletal muscle. Recovery from placentome was not attempted. Viral antigen was detected only in brain and foetal membranes. Gross abnormalities are illustrated in Plate 5.2.d, and consisted of diminished size of the hindquarters, hindlimb flexion and cutaneous haemorrhages. Moderately severe necrotic lesions were evident microscopically in cerebral hemispheres and mid-brain, and focal necrosis of skeletal muscle

fibres had also occurred. Foetus #53 was also examined at 63 days after a maximum of 31 days' exposure. Virus could not be recovered from the brain of this foetus, although viral antigen was detected in this tissue. Viable virus was recovered from placentome, foetal membranes, skeletal muscle, and allantoic fluid. Viral antigen was detected in brain, placentome, skeletal muscle and liver. The foetus showed no gross abnormalities, and the only tissue examined microscopically, namely placentome, was also normal. Foetus #49 was recovered at 66 days, after a maximum of 35 days' exposure. Viable virus was detected only in brain while no tissues were examined for viral antigen. Morphology was superficially normal, but there was severe histological damage. Focal necrosis and cavitation were visible in the intermediate zone and the ependyma of the cerebral hemispheres, focal necrosis was visible in skeletal muscle and a small number of placental trophoblast and syncytial cells were necrotic. Hypertrophy of the lymphoid component of the spleen had also occurred, while there had been a decrease in the number of cortical thymocytes. Ewe #40 had twins which were examined at 67 days, after a maximum of 27 days' exposure. One of these twins (#40A) was examined only for morphological changes. No gross changes were visible, but moderately severe necrotic lesions of the cerebral hemispheres, decreased quantity of thyroid colloid and increased splenic lymphoid development were visible microscopically. In the case of the other twin (#40B), both viable virus and viral antigen were detected only in brain and foetal membranes. The superficial morphology was normal, but characteristic necrotic lesions of moderate

severity were microscopically visible in cerebral hemispheres and skeletal muscle, while mild focal necrosis of placental trophoblast was seen. Foetus #50 was recovered at 69 days, after a maximum of 37 days' exposure. Viable virus was present in brain and foetal membranes while viral antigen was detected in brain, placentome and foetal membranes. Gross morphology was normal. Histological lesions were mild, and consisted of meningeal hyperaemia and characteristic necrosis in cerebral hemispheres and skeletal muscle. Foetus #46 was also examined at 69 days after a maximum of 38 days' exposure. Brain and spinal cord were totally absent, and skeletal muscle was not examined for the presence of viable virus or viral antigen. Of the remaining tissues, viable virus was recovered only from foetal membrane. Viral antigen was present in placentome, foetal membranes, spleen and thymus. Very severe gross and histological abnormalities were present. The gross abnormalities are illustrated in Plate 5.2.f, and consisted of diminished size of the hindquarters, arthrogryposis, kyphosis, brachygnathism, micrencephaly, cutaneous haemorrhages, subcutaneous oedema, abdominal distension and very poor circulation. Histologically, brain and spinal cord were absent, skeletal muscle contained foci of necrosis, focal haemorrhage and replacement by fibrous tissue, while focal necrosis of placental trophoblast and syncytium had occurred. Lymphoid development of the spleen was increased, the amount of thyroid colloid was decreased, and focal haemorrhages were visible in skeletal and cardiac muscle, lung and foetal membranes.

Foetus #33 was examined at 71 days, after a maximum of 27 days' exposure. This was the only foetus in this series to be

infected after 40 days of age. Viable virus was detected in placentome, foetal membranes, blood and allantoic fluid. Brain was not assayed for viable virus. Viral antigen was detected in brain, placentome, and skeletal muscle. The foetus was superficially normal, but severe microscopical lesions were seen. Focal necrosis was evident in all zones of the cerebral hemispheres, mid-brain, skeletal muscle and placental trophoblast and syncytium. In both brain and skeletal muscle necrosis was accompanied by focal haemorrhage and perivascular cuffing with mononuclear cells. Some fibrous tissue replacement of muscle fibres had occurred. Severe meningitis was present, involving hyperaemia and diffuse infiltration by mononuclear cells.

Ewe #36 had twins which were examined at 73 days, after a maximum of 33 days' exposure. One of these (#36A) was examined only for morphological changes. Its superficial abnormalities were minor, being restricted to reduction in size of the hindquarters. Microscopic examination revealed characteristic focal necrosis of cerebrum, mid-brain, cerebellum, and skeletal muscle. The meninges were hyperaemic and oedematous, and hyperaemia was also observed in thyroid, liver, lung and kidney tissue. The thyroid follicles contained less colloid than normal, and there were fewer cells in the thymic cortex. In the case of the other twin, viable virus was found in brain, placentome, foetal membranes and skeletal muscle, while viral antigen was detected only in the brain. This twin also had mild macroscopic abnormalities, namely subcutaneous oedema, diminished size of the hindquarters, and injected superficial cerebral blood vessels. Microscopically, the meninges were

hyperaemic, characteristic necrotic lesions were present in cerebrum and skeletal muscle, and fibrous tissue replacement of some muscle fibres had occurred. Ewe #37 also had twins which were examined at 75 days, after a maximum of 35 days' exposure. Twin #37A showed mild macroscopic changes, consisting of decreased hindquarter size, but was not examined further. Viable virus was recovered from brain, placentome, foetal membranes, and skeletal muscle of twin #37B. Viral antigen was detected in brain, placentome and foetal membranes. The foetus was normal on gross examination, and the only microscopic lesions detected were meningeal hyperaemia, and the usual focal necrotic lesions of cerebrum, mid-brain, skeletal muscle and placental trophoblast in a mild form. There was evidence also of increased development of the lymphoid component of the spleen. The final foetus, #S46, was also examined at 75 days, but had had a maximum of 44 days' exposure. Viable virus was detected in placentome and skeletal muscle, and viral antigen in brain, skeletal muscle and liver. Placentome was not examined for viral antigen. This foetus was grossly and histologically normal except for mild focal necrosis of skeletal muscle.

Distribution of virus

Thus, examination of the foetus for Akabane virus titre and the distribution of viral antigen revealed the presence of virus in at least one organ in all but two instances (#21 and #24, Table 5.1.1). Viable Akabane virus was most commonly recovered from placentome (74%), brain (65%) and foetal membrane (65%) over the whole age range tested. Virus was also isolated from skeletal muscle in some 50 per cent and from

foetal fluids in 30 per cent of foetuses. Successful isolation from the blood was rare (two cases out of 11), and no isolations were made from liver, spleen or thymus.

Although viral antigen was more consistently demonstrated using fluorescent microscopy in brain, placentome, foetal membrane and skeletal muscle (Table 5.2), neither the range of tissues in which, nor the stage of gestation at which it could be detected was extended beyond that revealed by virus titrations. Examination of foetal tissues, after staining with fluorescent antibody, revealed a distribution of Akabane antigen consonant with the distribution of histopathological changes (see Table 5.3). Viral antigen was detected in the brain in 18 out of 28 foetuses examined. The rate of detection was slightly lower in placentomes. Approximately 50 per cent of the foetuses exhibited fluorescence in foetal membranes and a similar proportion did so in skeletal muscle. Only four out of 28 foetuses (examined over a wide scatter of ages), had fluorescence in the liver whilst there was only one instance of fluorescence in spleen or thymus. Both of these organs labelled positively in that foetus which exhibited the most severe histopathological changes, but only scattered cells were involved.

Plate 5.3 demonstrates the occurrence of positive fluorescent staining of tissues from infected foetuses. By comparison of stained cryosections with haematoxylin-eosin stained paraffin sections of the relevant foetal tissue, it was possible to draw some inferences about the nature of the cells in which the viral antigen was localized. Antigen was detected

PLATE 5.3 Fluorescein-labelled Akabane antigen in tissues of transplacentally infected foetuses.

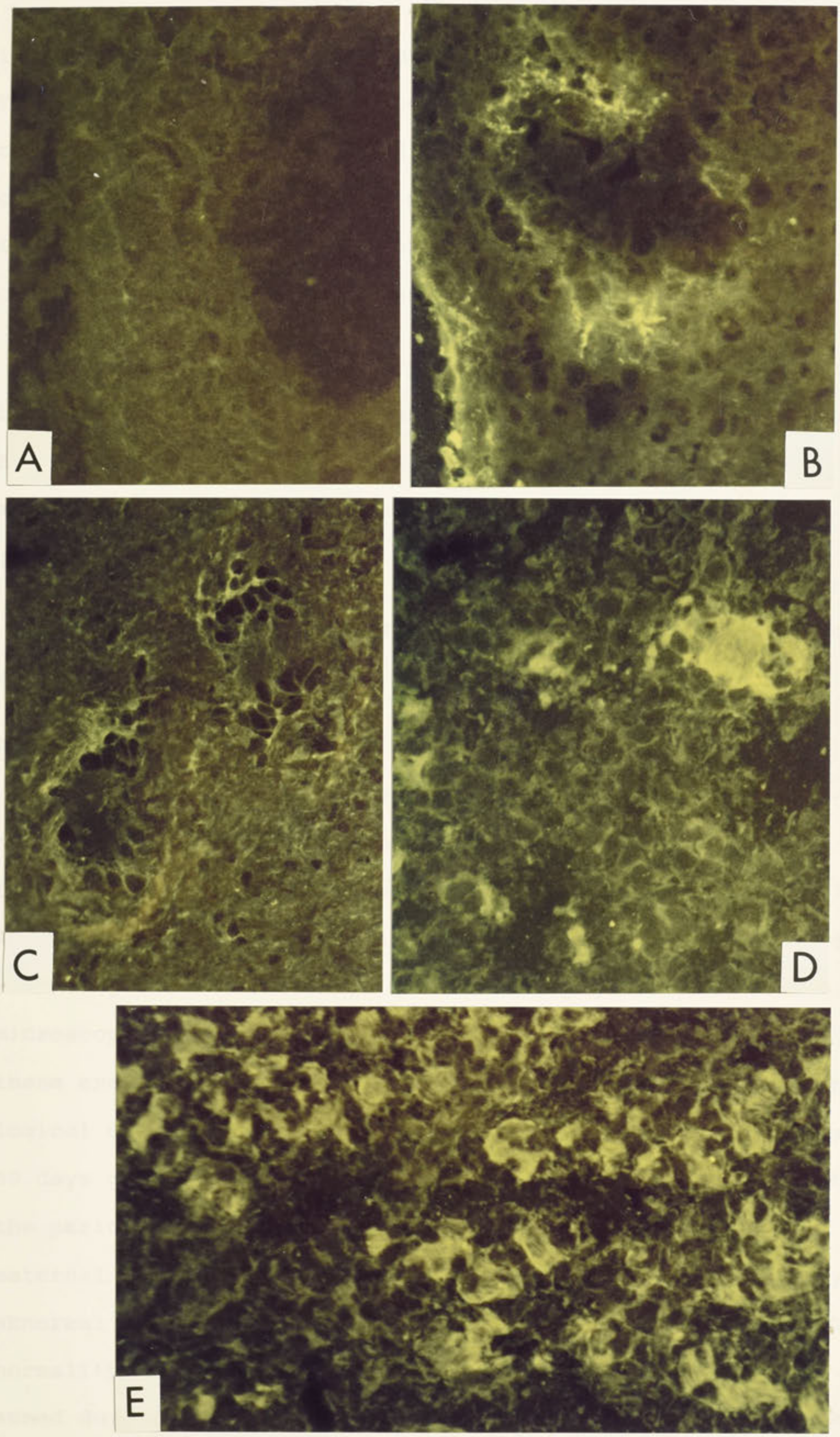
A: #43, aged 55d, placentome, control stained section.
x 250.

B: #43, aged 55d, placentome, 'test' stained section. Specific fluorescein labelling occurred in the cytoplasm of foetal trophoblast cells. Not all trophoblast cells in a villus were labelled.
x 250.

C: #53, aged 63d, placentome. Specific fluorescein labelling occurred in the cytoplasm of foetal trophoblast cells.
x 250.

D: #43, aged 55d, skeletal muscle. Specific fluorescein labelling occurred in the cytoplasm of scattered myocytes and of groups of myocytes.
x 250.

E: #25, aged 45d, skeletal muscle. Widespread specific fluorescein labelling occurred in the sarcoplasm.
x 250.


A
B
C
D
E

in small groups of skeletal muscle fibres (Plate 5.3, D & E). Foetal trophoblast appeared to be the component of placentomes that was principally affected (Plate 5.3, B & C). Antigen could not be detected in the maternal tissue of the placentome. In foetal membranes, the fluorescence appeared in trophoblast cells, and occasionally in vascular endothelium.

A wide variety of cell types in the brain stained positively including neurones, neuroglia and vascular endothelium. Both scattered cells and small groups of cells were involved, and cells adjacent to lesions (e.g. cavitations) were frequently positive.

In all tissues, fluorescence was restricted to the cytoplasm of cells.

Pathological changes

No instance of gross or microscopic abnormality was observed in morphological examination of any of the control foetuses. Eight of the first nine foetuses recovered (at 37-49 days), following maternal infection at 31-33 days, had no morphological abnormalities detectable by gross or optical microscopic examination. Virus was recovered from all of these except #21 and #24. The youngest foetus in which pathological changes were macroscopically evident was recovered at 50 days gestation (16 days post maternal inoculation). Within the period of 50 days to 75 days gestation (16-44 days post maternal inoculation), eight out of 23 foetuses were grossly abnormal (Tables 5.3.1 and 5.3.2). The presence of gross abnormality appeared not to be related to foetal age or presumed duration of exposure to virus. Nevertheless there was

a progression in the types of abnormality observed. Younger foetuses with a concomitantly shorter exposure before autopsy sustained a decrease in the size of cranium and hindquarters (Plate 5.2, b and d). As age and exposure time increased, arthrogryposis, kyphosis and subcutaneous oedema also appeared. Only one foetus (69 days) showed severe pathological changes (Plate 5.2, f). In addition to the preceding features, there were cutaneous petechial haemorrhages, and haemorrhages in skeletal muscle, lung and thymus, together with an increase in haemorrhagic peritoneal fluid, very poor circulation, reduction in amniotic and allantoic fluids and a total absence of brain and spinal cord. In summary, macroscopically apparent lesions were present in foetuses inoculated between 31 and 40 days gestation, and recovered between 50 and 75 days gestation.

Using microscopic examination, the earliest detection of abnormality was in a 41 day foetus (10 days after maternal inoculation). The abnormalities at this stage were confined to malacia and focal necrosis with cavitation of mid-brain and cerebrum. The five next oldest foetuses (44 to 49 days) were normal, and thereafter, that is from 50 days on and after a minimum of 16 days' exposure to virus, all lambs (21) showed histopathological changes. The brain was always involved with occurrence of focal necrosis, malacia and cavitation in the cerebral hemispheres, especially in the subcortical region but sometimes also in ependyma and mid-brain (Plate 6.2, D). Foci of necrosis were characterized by vacuolation, karyolysis and pyknosis of nuclei with loss of cytoplasmic processes of all cell types. Occasionally, hyperaemia and focal haemorrhage were observed. The cerebellum was normal in all five instances

in which it was examined. Brain lesions were more severe in the older foetuses, but only one foetus (#33, 71 days) showed evidence of an inflammatory cell reaction with infiltration of mononuclear cells into meninges and brain, and perivascular cuffing.

The meninges were involved in most foetuses at, or older than, 67 days with hyperaemia and, occasionally, increased cellularity. Thorough examination of the spinal cord of two animals which had histologically detectable brain lesions did not reveal any abnormalities.

Seven out of 13 foetuses at or above 56 days gestation showed a decreased number of cortical thymocytes (a decrease of approximately 20% in the cells per high power field) and eight out of 11 (even age distribution 56-75 days) showed a decreased amount of colloid in the thyroid follicles. This decrease in colloid was apparently evenly distributed among follicles throughout the thyroid. No abnormalities were observed in liver, lung, heart or adrenal. The only kidney lesion to be detected was in #33 in which multiple cysts and focal haemorrhage were observed. Three out of 21 foetuses had early perivascular cuffing in the foetal membranes, and four out of 21 foetuses had oedematous foetal membranes. All of these animals were at least 69 days old. A small number of the foetuses showed necrosis of occasional placental trophoblast cells.

Skeletal muscle abnormality was first observed in a 52 day foetus and was thereafter detected in 18 out of 20 foetuses. It comprised foci of necrosis together with

fibrosis. Swelling and vacuolation of cytoplasm and pyknosis resulted in a decrease in the number and diameter of fibres (A similar lesion is shown in Plate 6.6,C). Sarcolemma and nuclei often remained after loss of sarcoplasm producing an appearance which simulated adipose tissue replacement. The necrosis was occasionally accompanied by focal haemorrhages, but there was no cellular reaction or infiltration observed with the exception of one 71 day-old lamb (#33) which had early perivascular cuffing.

In summary, histopathological lesions were present in foetuses over the whole age range at inoculation (31-44 days). The salient features were necrosis of brain and skeletal muscle from ten days post maternal infection, sometimes accompanied by focal haemorrhage, with the severity of these lesions increasing with age. Increased development of the splenic white pulp and decreased production of colloid by the thyroid was apparent from 56 days, while there was a decreased number of cortical thymocytes in some older foetuses (66 days). Three foetuses older than 68 days had cellular infiltration of brain, skeletal muscle and/or foetal membranes.

Virus-specific antibody

Eighteen of the 20 infected ewes tested for specific antibody were shown to have seroconverted either by seven days after infection, or when first tested where this was more than seven days after infection. These results are summarized in Table5.4. Specific neutralizing antibody was not detected in serum or foetal fluid from the 14 control ewes and their foetuses, nor from any of the 15 infected foetuses tested

TABLE 5.4 - Specific neutralizing antibody levels in serum of pregnant ewes after I/V injection of Akabane virus.

	Time after inoculation (d)				
Ewe	0	7	14	21	28
30	0	10			
22	0	0			
29	0	0			
21	0	11			
51	0	15			
39	0		18	17	
45	0	12	19	19	
43	0		18	14	14
32	0	14	18	17	
28	0	16	19	17	
30B	0	15	17	13	15
26	0	16	15	15	
31	0	15	14	18	17
53	0			12	13
49	0	15	18	14	12
40B	0	10	14	16	14
46	0	14	19	14	17
36B	0	18	21		13
33	0	14	12		11
37	0	11	19		11

Antibody level was assessed by the plaque inhibition assay, and is expressed as the mean diameter (mm) of the zone of inhibition in duplicate test plates.

(Table 5.5), despite the fact that three of these were aged 75 days.

In vitro transformation assays

The results of the in vitro transformation assays of liver, thymus and spleen cells using mitogens and Akabane virus as activators are summarized in Tables 5.6 and 5.7. Liver cells from 14 control foetuses between 39 and 76 days were assayed, as were thymus cells from four of these foetuses and spleen cells from one. There was considerable variability among the results of control foetal liver cell reactivity (Table 5.6). Nevertheless responses to PHA and LPS were significantly above the background level with cells from two of the four foetuses aged between 49 and 62 days. With one exception, responses of control liver cells to Con A did not exceed background levels. The exception was the 62 day foetus which also responded to PHA and LPS. There was no evidence of any specific responses to Akabane virus when adjustment was made for proliferation induced by Vero cells alone. Spleen cells from one foetus were assayed at 75 days, and gave a good response to Con A but none to Akabane virus. Thymus cells from four foetuses were assayed at 71-76 days and also gave very good responses to Con A but none to virus.

The in vitro responses to mitogens of liver cells from Akabane-infected foetuses are shown in Table 5.7. Liver cells from 13 foetuses aged between 38 and 75 days were assayed for response to Akabane virus, to Con A, and to at least one other mitogen (PHA, LPS or PWM). In contrast with the capacity, albeit inconstant, of liver cells from normal foetuses to respond to PHA and LPS (Table 5.6), cells from Akabane-infected

TABLE 5.5 - Specific neutralizing antibody titre in foetal sera after transplacental infection with Akabane virus

Foetus	Age inoculated (d)	Age recovered (d)	Antibody titre
39	31	52	0
S32	31	55	0
32	34	56	0
28	33	56	0
30B	32	59	0
26	34	62	0
31	31	63	0
49	31	66	0
40B	40	67	0
50	32	69	0
36B	40	73	0
33	44	71	0
37A	40	75	0
37B	40	75	0
S46	31	75	0

TABLE 5.6 - Response (^{3}H-thymidine incorporation) of cells from control foetuses to mitogens and antigens.

Cell type	Foetus No.	Foetus Age(d)	Controls: Medium Cpm	Controls: Vero Cpm	Non-specific stimulation: Con A Cpm	Con A SI	PHA Cpm	PHA SI	LPS Cpm	LPS SI	PWM Cpm	PWM SI	Specific stimulation: Akabane Cpm	Akabane SI	Inactive Akabane[+] Cpm	Inactive Akabane[+] SI
Liver	82	39	451	434	313	0.7							394	0.9	129	0.3
	151	40	3000		1789	0.6	3040	1.0	2769	0.9						
	173	41	6374		1791	0.3			5774	1.0						
	94	47	638	506	806	1.3							371	0.7	398	0.8
	43	49	1706	2591	2253	1.3	341	0.2			927	0.5	1520	0.6	1415	0.5
	53	49	3181		1114	0.4	14418	4.5	9174	2.9						
	141	50	2605		3030	1.2	2614	1.0	4221	1.6						
	83	60	1471	713	905	0.6							488	0.7		
	44	62	521	862	1833	3.5	1462	2.8	1174	2.3	1034	2.0	968	1.1	901	1.0
	102	71	297	378	490	1.6							257	0.7	186	0.5
	89	75	1560	416	927	0.6							485	1.2	811	1.9
	38	75	742	1202	508	0.7	695	0.9	1208	1.6	606	0.8	1259	1.0	1480	1.2
	88A	76	570	134	403	0.7							159	1.2	292	2.2
	88B	76	180	492	148	0.8							291	0.6	123	0.3
Thymus	102	71	3137	729	50840	16.2							1330	1.8	321	0.4
	89	75	3222	3318	34889	10.8							2740	0.8	1123	0.3
	88A	76	2143	661	79972	37.3							665	1.0		
	88B	76	646	264	12614	19.5							288	1.1	133	0.5
Spleen	89	75	4849	5138	79924	16.5							4002	0.8	3236	0.3

Cpm = counts per minute of β emission from incorporated thymidine.

SI = stimulation index = $\frac{\text{Cpm of stimulated cultures}}{\text{Cpm of control* cultures}}$

* Cultures with medium only were used as controls for mitogens Con A, PHA, LPS and PWM.

Cultures with Vero antigen were used as controls for Akabane virus.

[+] Inactive Akabane = Akabane virus that has been inactivated by U/V irradiation before use as a stimulator.

TABLE 5.7 - Response (^{3}H-thymidine incorporation) of liver cells from foetuses transplacentally infected with Akabane virus to mitogens and antigens.

			Controls			Non-specific stimulation							Specific stimulation	
Foetus	Age (d) at inoculation	Age (d) at assay	Medium Cpm	Vero Cpm	Con A Cpm	SI	PHA Cpm	SI	LPS Cpm	SI	PWM Cpm	SI	Akabane Cpm	SI
S30	31	38	5590	6473	3287	0.6			2042	0.4	2033	0.4	4999	0.8
Y22	31	40	1039	798	2050	2.0	1520	1.5			996	1.0	1285	1.6
S25	33	45	2246	1521	1336	0.5	1265	0.5			1760	0.7	3646	2.4
39	31	52	1343	1331	839	0.6			524	0.4			1024	0.8
43	32	55	173	216	396	2.3	148	1.0	109	0.6			552	2.6
S32	31	55	4217	3769	1843	0.4	917	0.2			1538	0.4	3827	1.0
26	34	62	7761	6958	2752	0.4	4330	0.6	699	0.1			4667	0.7
40	40	67	5020	6538					996	0.2			7860	1.2
46	31	69	1309	1342	607	0.5			854	0.7			1401	1.0
50	32	69	389	261	76	0.2	68	0.2	87	0.2			573	2.2
33	44	75	2137	622	130	0.1	1471	0.7	3642	1.7			154	0.3
37	40	75	379	127	128	0.3	265	0.7	305	0.8			299	2.4
S46	40	75	2013	2118	1746	0.9	1237	0.6	1930	1.0			1867	0.9

Cpm = counts per minute of β emission from incorporated thymidine.

SI = stimulation index = $\frac{\text{Cpm of stimulated cultures}}{\text{Cpm of control* cultures}}$

* Cultures with medium were used as controls for mitogens Con A, PHA, LPS and PWM. Cultures with Vero were used as controls for Akabane virus.

foetuses uniformly failed to respond to PHA, LPS or PWM, and cells from only one foetus (aged 55 days) responded marginally to Con A. This failure to respond to mitogens did not become less evident with maturation of the subject. In contrast with this failure to respond to non-specific mitogens, liver cells from four of the 13 infected foetuses, evenly distributed in age between 45 and 75 days, gave responses to Akabane virus slightly exceeding those to Vero antigen (Table 5.7).

5.4 DISCUSSION

Infection of pre-implantation embryos by injection of virus into the ewe was not demonstrated. Although the time required for the virus to cross the established placenta after maternal inoculation is less than 24 hours (Parsonson, personal communication), four days may have been insufficient in the absence of a placenta. Alternatively, the 18 to 22 day embryo may have been incapable of supporting survival or replication of the virus after entry, because of the cell and tissue immaturity. In this event the virus, although present, may not have been detected because of the high sampling error of the technique used. The third possibility is that Akabane virus is incapable of transmission from mother to embryo in the absence of a placental junction.

While the cells of the foetal brain did not incorporate radio-labelled thymidine, it is unlikely that this resulted from lack of mitotic activity in this organ. It is more probable that the thymidine did not reach the brain because it was incorporated in other tissues first, or because the blood/brain barrier effect prevented its uptake by neural cells.

The latter is unlikely, however, as thymidine is a very small molecule and its uptake by neural cells of guinea-pigs has been demonstrated (Wanner, 1975).

With the exception of skeletal muscle, there does not appear to be any correlation between occurrence of active division at the time of recovery, and the occurrence of severe pathological changes or high viral titres and viral antigen levels at recovery. Although Parsonson et al, (1981b) considered that the ability of the virus to reach rapidly dividing cells may determine the degree and extent of the damage to the foetus, it appears from these results that rapid cell division is not the sole factor determining either viral persistence or tissue damage.

Despite the presence of histopathological lesions in most of these foetuses, only a small proportion exhibited gross damage and in only one individual (#46) was the macroscopic pathology severe. It is possible that severe deformities only developed when removal of the foetus is left until nearer to term, as Parsonson et al, (1981a) found 80 per cent to be grossly abnormal when autopsied at 69-106 days.

The heavy infection of placentomes (foetal trophoblast in particular) was only rarely associated with placental lesions and these were mild. The virus may not be very cytopathic for placental cells, or it may be that the foetal trophoblast regenerates rapidly, and lesions therefore do not develop to the stage of being readily detectable. It appears highly likely that the period of exposure of the foetal lamb to Akabane virus after maternal infection is much more pro-

longed than the transient viraemia in the ewe might suggest. Having reported persistence of virus in placentomes through much of pregnancy, Parsonson et al, (1981b) speculated that its prolonged release from this site throughout this time was likely. Despite this, it appears likely that the period over which infection of the pregnant ewe may lead to detectable foetal abnormality is more limited (Narita et al, 1979).

The changes observed in skeletal muscle resembled those previously found in Akabane-infected foetuses (Parsonson et al, 1981b) except that connective tissue or adipose tissue replacement of necrotic myocytes was not observed.

The cystic cavities observed in the cerebral hemispheres of these foetuses resembled those previously reported in foetuses infected at 30-36 days (Parsonson et al, 1981b); however, the perivascular cuffing observed in some of their foetuses was found in only one of these - the eldest at infection (44 days). The mineralization and gliosis of the cerebrum and pons reported by these authors were not seen in foetuses in this study. The conclusion, drawn from histological findings, that the virus infection was directly responsible for the encephalopathic lesions of foetal lambs with Akabane disease (Narita et al, 1979) is confirmed by these histological and virological studies.

The histological and fluorescent evidence suggested that the virus had produced primary cell necrosis, and had shown tropism for trophoblast, brain and skeletal muscle (and, in view of the focal haemorrhage, possibly for vascular endothelium). The occurrence of mononuclear cell infiltration in the

one foetus may have led to further and faster tissue destruction. It was not, however, invariably associated with occurrence of foetal pathology. Skeletal muscle abnormalities appeared not to be dependent solely on degeneration secondary to neuropathological changes, as suggested by Hartley et al, (1977) but were contributed to by viral necrosis of skeletal myocytes.

Cellular immunological reactivity was examined using liver cells from foetuses between 38 and 75 days gestation. The morphological nature of the cells prepared from the liver over this period would be expected to have changed as haemopoiesis decreased in this organ. That failure of the liver cells of these infected foetuses to respond to any of the stimuli utilized remained evident with maturation of the subject may thus be attributable to a progressive decrease in the frequency of immunocompetent cells in liver of the older foetuses or to a consistently low frequency of these cells in liver throughout foetal development.

The results suggest that Akabane infection may non-specifically depress the response of foetal liver cells to mitogens. However, the inconstancy and the low levels of responses to mitogens often observed in normal foetuses necessitate some caution in characterizing the responses of the infected group as depressed. Depression of responsiveness could be accounted for by the demonstrated persistence of virus in the infected foetuses (see Table 5.1).

There was no indication that the 15 foetuses examined after being infected transplacentally at days 31 to 44 were

capable of mounting a humoral response by 75 days gestation. No transplacentally exposed foetuses were examined after this time, so it is not known whether these foetuses would have developed a humoral response. In contrast with these results, specific humoral responses have been observed in foetuses of 64 and 75 days which had been transplacentally infected with Akabane virus between 32 and 36 days (Parsonson et al, 1981a; Hashiguchi et al, 1979). Despite the lack of humoral response, four of the foetuses in this study gave slight antigen-specific in vitro cellular responses which may indicate some cellular response.

In summary, some aspects of Akabane viral behaviour and host response were studied in foetuses of ewes infected between 18 and 44 days gestation and examined four to 44 days later when the embryos or foetuses were 22 to 75 days of age. One group of ewes was infected via the uterine artery with 5.0×10^7 PFU of viable Akabane virus on day 18, that is before implantation, and the embryos were examined four days later for the presence of Akabane virions. There was no evidence that these foetuses became infected.

The 28 ewes of the second group were infected via the jugular vein with 10^7 PFU of viable Akabane virus between 31 and 44 days gestation, i.e. after implantation. Fourteen of their foetuses were injected with ^{3}H-thymidine before being removed and autoradiographed. The label was chiefly taken up by the haemopoietic cells of the liver, and to a lesser degree by the myocytes of the skeletal muscle and the heart, suggesting that these cells were dividing rapidly. Twenty-eight

foetuses were examined for distribution and titre of virus. Virus was most consistently detected in placentome, chorioallantoic membrane, brain and skeletal muscle. The highest viral titres occurred in placentome and chorioallantoic membrane. Thirty foetuses were examined for pathological changes. These were consistently present in foetuses of 50 days and older, and involved principally cerebrum and skeletal muscle. Only one of nine foetuses younger than 50 days was affected.

Liver cells from 13 foetuses aged from 38 to 75 days were assayed for in vitro transformation responses to live Akabane virus and to mitogens. Only one responded to mitogen (55 days), and four (45, 55, 69 and 75 days) responded slightly to Akabane virus. Serum or allantoic fluid of 15 foetuses (aged from 52 to 75 days) was assayed for specific antibody, and all results were negative. Eighteen of the 20 ewes whose sera were similarly assayed all seroconverted when first tested, usually seven days after inoculation.

In conclusion, foetuses were transplacentally infected at 31 to 44 days, with virus localizing chiefly in placentome, brain and skeletal muscle. This was accompanied by focal necrosis which was most marked in brain and skeletal muscle, and in the case of the foetus infected at 44 days also by focal mononuclear cell infiltration of brain. There was no evidence of specific antibody production in these foetuses inoculated by day 45 and examined by day 75, although there was evidence for an inconsistent specific in vitro lymphocyte response. There was no evidence that infection of cells or morphological lesions were limited to rapidly dividing tissues.

CHAPTER 6

DIRECT SYSTEMIC AKABANE VIRAL INFECTION OF THE SHEEP FOETUS

CHAPTER 6 - DIRECT SYSTEMIC AKABANE VIRAL INFECTION OF THE SHEEP FOETUS

6.1 INTRODUCTION

Akabane virus is able to cross the placenta as late as 101 days gestation, but foetuses infected at this age do not develop lesions (Narita *et al*, 1979; Hashiguchi *et al*, 1979). It is not possible to decide with certainty whether this absence of lesions reflects the passage of insufficient virus across the placenta or an increase in foetal resistance. Increased resistance could conceivably be due either to intervention of an immune response, or to a relative target organ insusceptibility. One means of simplifying interpretation of this situation was to examine the effect of direct exposure of the foetus to a substantial inoculation of virus. Akabane virus was therefore injected directly into the foetus *in utero*, thereby bypassing the placenta. It was hoped that comparison of the development of the infection in the directly inoculated foetus with that in the foetus infected transplacentally at a variety of gestational ages would provide some indication of the significance of the placenta, in regulating access of the virus to the foetus. It also ensured that each foetus received the same dose of virus. Concurrently these experiments were intended to determine the extent to which the type and severity of tissue damage varied with the route of infection, foetal age at the time of infection, or duration of exposure of the foetus to the virus.

Two methods of direct foetal challenge were used. In the first, foetuses were systemically challenged by intravenous

or intraperitoneal injection of Akabane virus and a single time point measurement of response was made when the foetus was recovered. The results are presented in this chapter. In the second, older foetuses were locally challenged, and measurements of the response were made at intervals thereafter. The results of this procedure will be presented in Chapter Seven.

6.2 EXPERIMENTAL DESIGN

Seventeen foetuses aged between 38 and 120 days gestation were directly inoculated with 0.2 ml of medium containing 2.0 x 10^6 PFU Akabane virus and recovered for examination five to 14 days later at 43 to 134 days gestation, as described in Materials and Methods. The first three foetuses were inoculated via the umbilical vein at 38 to 45 days. All three died and, consequently, subsequent inoculations were made I/P with only two ensuing deaths (in foetuses inoculated at day 70 and day 89).

The 12 infected foetuses surviving until the time of removal were examined as described in Materials and Methods, for the presence of gross and microscopic signs of disease, the distribution and titre of virus, and the immunological response. Whenever the foetus was sufficiently large for a serum sample to be collected, this was tested for the presence of specific neutralizing antibody. Amniotic and allantoic fluids were also similarly tested when available. The *in vitro* reactivity of the liver, spleen and thymus cells were determined by LTA, using non-specific (Con A) and specific (Akabane antigen) activators.

Fifteen non-inoculated foetuses were recovered at intervals between 39 and 76 days gestation for examination as controls. The same animals were used as controls for the studies described in Chapter Five.

Eleven of the ewes with infected foetuses, and all of the control ewes, were bled at the time of foetal recovery and the sera were tested for specific neutralizing antibody.

6.3 RESULTS

The results of examinations of foetuses for viral titre and distribution of Akabane viral antigen æ shown in Tables 6.1 and 6.2. Neither virus nor antigen was detected in the tissues of any of the 15 control foetuses, and these results are therefore not tabulated. For each foetus, samples of three placentomes were taken. Only one of these was examined for viral antigen. Initially the three were separately assayed for viable virus, but when the variability of titre became obvious the three samples were pooled and a single assay performed on the combined samples. The pathological lesions observed in infected foetuses are summarized in Table 6.3, and the results of assay for specific antibody are tabulated in Table 6.4.

Viable virus could not be isolated from any tissues other than placentome of foetuses older than 100 days at autopsy, despite the fact that a substantial number of tissues from these foetuses was examined.

The three youngest foetuses to be inoculated had died by the time of recovery and consequently no samples were obtained.

TABLE 6.1.1 - Distribution of viable Akabane virus in foetuses following direct intraperitoneal or intravenous inoculation

Sheep	1[a,b]	2[a,b]	3[a,b]	45	44	39	24	35	23	38	85[a]
Age(d) at inoculation	38	45	45	50	50	55	55	60	60	60	70
Age(d) at recovery	43	50	59	58	65	60	70	65	71	75	84
Virus titre (PFU/ml):											
Cerebrum				$1.0x10^4$	$4.5x10^2$	$5.0x10^6$	$1.5x10^2$	$1.5x10^4$	$1.0x10^2$	+	
Placentome				+	$1.5x10^3$	30	15	$2.5x10^2$	$5.0x10^4$	$4.0x10^3$	$2.5x10^3$
Allantoic membrane				$1.7x10^4$	$5.0x10^2$	15	+	$2.0x10^2$	$1.0x10^2$		
Amniotic membrane						20	25	$2.1x10^4$	$1.3x10^4$		
Skeletal muscle				$1.0x10^2$	$1.5x10^2$	$1.5x10^2$		30	10	-	
Spleen					-		-	-	-		
Thymus				-	-		-		-		
Lung				-	-						
Blood					+	10		+	-	-	
Allantoic fluid				$3.5x10^2$	$1.5x10^4$		-	-	$2.3x10^3$	$5.0x10^2$	
Amniotic fluid				$3.1x10^4$	$1.1x10^4$		25		$2.5x10^4$		
Liver				$2.3x10^3$							

+ = cytopathic effect, - = no cytopathic effect or titre.

Virus titre in tissues is expressed as PFU/ml of the 10% suspension (see Materials and Methods).

a = foetus was dead when recovered; b = infected by I/V injection.

TABLE 6.1.2 - Distribution of viable Akabane virus in foetuses following direct intraperitoneal inoculation, continued.

Sheep	81	97	92[a]	77	104	105
Age(d) at inoculation	80	80	89	89	100	120
Age at recovery	89	94	103	103	114	134
Virus titre (PFU/ml):						
Cerebrum	$2.0x10^2$	+		-	-	-
Placentome	$1.9x10^3$	+	$6.5x10^2$	25	$5.0x10^2$	$3.4x10^3$
Allantoic membrane	$2.0x10^4$	-		-	-	-
Amniotic membrane	$3.5x10^4$					
Skeletal muscle	+	-		-	-	-
Spleen		15		-	-	-
Thymus				-	-	-
Lung	15	-			-	
Spinal cord		-		-	-	-
Blood	5	-		-	-	-
Allantoic fluid	75	-		-	-	-
Amniotic fluid		-		-	-	-
Liver	-	-		-	-	-

+ = cytopathic effect; - = no cytopathic effect or titre.

Virus titre in tissues is expressed as PFU/ml of the 10% suspension.

a = foetus was dead when retrieved.

TABLE 6.2 - Distribution of Akabane viral antigen in foetuses following direct intraperitoneal or intravenous inoculation

Sheep	1[a,b]	2[a,b]	3[a,b]	45	44	39	24	35	23	38	85[a]	81	97	92[a]	77	104	105
Age(d) at inoculation	38	45	45	50	50	55	55	60	60	60	70	80	80	89	89	100	120
Age(d) at recovery	43	50	59	58	65	60	70	65	71	75	84	89	94	103	103	114	134
Stained antigen:																	
Cerebrum				+			+	+	+	-		+	+		+	+	+
Placentome				+	-	+	+	+	+	-		+	+			+	
Skeletal muscle				+	-	-	+	-	-	-			-		-	+	
Foetal membrane					-	-	-	+	+	-		+	-		-	+	
Liver				+	-	-	+	-	-	-							
Spleen				-		-	-	-	-	-							
Thymus				-	-	-	-	-	-	-						-	
Spinal cord													+			+	

The antigen was detected by specific immunofluorescent staining.

+ = antigen present; - = antigen not detected.

a = foetus was dead when recovered; b = infected by I/V injection.

TABLE 6.3 - Gross and histological abnormalities of foetuses directly infected by intraperitoneal or intravenous injection of Akabane virus

Sheep	1[a,b]	2[a,b]	3[a,b]	45	44	39	24	35	23	38	85[a]	81	97	92[a]	77	104	105
Age(d) at inoculation	38	45	45	50	50	55	55	60	60	60	70	80	80	89	89	100	120
Age(d) at recovery	43	50	59	58	65	60	70	65	71	75	84	89	94	103	103	114	134
Gross Pathology				-	+	+	++	-	-	+++		++	-		-	-	-
Histopathology:																	
Cerebrum				++	+++	+	+++	+	+	+++		+++	+		+	+	+
Cerebellum							+	+		+		++	+		-	-	-
Spinal cord				+	-	+	+	-	-	+		++	-		-	-	+
Meninges				+++	+++	++	+++	+	++	+++		++	+		-	+	+
CNS infiltration				++	+++	+	+++	+	-	+++		++	+			-	+
Heart infiltration				-	-	-	+	-	-	+		-	-		-	-	-
Lung infiltration				+	-	-	-	-	-	+		-	-		-	-	-
Kidney infiltration				+	-	-	+	-	-	+		-	-		-	-	-
Membrane infiltration				-	-	-	+	-	-	+		+	-		-	-	+
Muscle infiltration				-	-	-	-	-	-	-		+	-		-	-	-
Placentome				+	++	+	+	+	-	+		++	-		-	+	+
Skeletal muscle				+	+	+	+	+	++	++		++	+		-	-	-
Foetal membrane						+	+	-				+	+		-	+	+

Abnormalities were scored (-) - (++++), where (++++) = total loss of tissue.

a = foetus was dead when recovered; b = infected by I/V injection.

TABLE 6.4 - Specific neutralizing antibody levels in serum and foetal fluid of foetuses directly infected by intraperitoneal injection of Akabane virus, and in sera of the ewes.

Ewe	Foetal age (d) at inoculation	Adult sera Time(d) after inoculation				Foetal sera Time(d) after inoculation				Foetal fluids Time(d) after inoculation			
		0	5	10	15	5	8	10	15	5	8	10	15
45	50						0				0		
44	50	0			20				0				0
39	55	0	8			0				0			
24	55	0			12				0				0
35	60	0				0							0
23	60	0		15				0				0	
38	60	0			15				8				0
85	80	0	0		21								9
81	80	0	0		7			0					
97	80	0	0		18				4				0
92	89	0											
77	89	0		15		5				0			
104	100	0		11		13				0			
105	120	0		16		11				0			

Antibody levels are expressed as the diameter (mm) of the zone of inhibition.

Foetal fluid = allantoic fluid.

Foetus #45 was recovered for examination at 58 days, eight days after it had been inoculated with live Akabane virus. Viable virus was recovered from cerebrum, placentome, allantoic membrane, skeletal nuscle, allantoic and amniotic fluid and liver. Viral antigen was detected in cerebrum, placentome, skeletal muscle and liver. The gross morphology was normal, but typical focal necrotic lesions were present in cerebral hemispheres, spinal cord, placentome and skeletal muscle. In addition, both focal and generalized infiltration by lymphocytes and monocytes was observed in the meninges, central nervous system tissues, lung and kidney. Specific anti-viral antibody could not be detected. Foetus #44 was examined at 65 days, 15 days after inoculation. Viable virus was recovered from the cerebrum, placentomes, allantoic membrane, skeletal muscle, blood, allantoic and amniotic fluid, but viral antigen could not be detected. Autopsy revealed micrencephaly, and severe histologically detectable lesions of brain, meninges, placentome and skeletal muscle which resembled those of #45. Foetus #39 was examined at 60 days, five days after inoculation. Virus was recovered from cerebrum, placentome, allantoic and amniotic membranes, skeletal muscle and blood, but viral antigen could only be demonstrated in placentomes. The volume of peritoneal fluid was abnormally high, and the usual necrotic and infiltrative lesions were present in cerebrum, spinal cord, meninges, placentome, skeletal muscle and foetal membranes. Specific antibody could not be detected. Foetus #24 was examined at 70 days, 15 days after inoculation. Viable virus was recovered from cerebrum, placentome, allantoic and amniotic membranes and amniotic fluid, and antigen was detected in cerebrum, placentome,

skeletal muscle and liver. The gross morphology was severely abnormal, the lesions being micrencephaly, fluid-filled cerebral hemispheres, and decreased size of the hindquarters. Characteristic severe lesions were present on histological examination of cerebrum and meninges, while mild lesions were noted in spinal cord, cerebellum, heart, kidney, foetal membrane, placentome and skeletal muscle. No specific antibody was detected. Foetus #35 was examined at 65 days, five days after inoculation. Viable virus was detected in cerebrum, placentome, skeletal muscle, allantoic and amniotic membranes and allantoic fluid. Viral antigen was present in brain, placentome and foetal membrane. Macroscopic abnormalities were not observed, although mild histological lesions were present in cerebrum, cerebellum, meninges, placentome and skeletal muscle. Specific antibody was not detected. Foetus #23 was examined at 71 days, 11 days after inoculation. Viable virus was present in cerebrum, placentome, allantoic and amniotic membranes and fluids and skeletal muscle. Viral antigen was detected only in cerebrum, placentome and foetal membrane. The foetus appeared to be grossly normal, although mild characteristic microscopical lesions were observed in cerebrum, meninges and skeletal muscle. Specific antibody was not detected.

Foetus #38 was examined at 75 days, 15 days after inoculation. Virus was recovered from cerebrum, placentome and allantoic fluid, although viral antigen was not detected in any tissue. Severe gross and microscopic abnormalities were observed, namely micrencephaly, fluid-filled cerebral hemispheres, decreased size of the hindquarters, and characteristic

lesions of cerebrum, cerebellum, spinal cord, meninges, placentome and skeletal muscle. This foetus was the youngest in which specific antibody was detected. Foetus #85, examined at 84 days after 14 days exposure to virus, was dead when recovered. Virus was recovered from placentome, but no other tissues were examined. Foetus #81 was recovered at 89 days, nine days after inoculation. Viable virus was present in cerebrum, placentome, skeletal muscle, lung, blood, allantoic fluid and allantoic and amniotic membranes. Viral antigen was detected in the three tissues tested, namely cerebrum, placentome and allantoic membrane. Moderately severe gross and histological lesions were observed. Brain abnormalities consisted of micrencephaly, hydranencephaly, meningitis, and necrosis and infiltration of cerebrum, cerebellum and spinal cord. Hindquarter muscle bulk was decreased, and necrosis and infiltration of skeletal muscle had occurred. Characteristic microscopic lesions were also evident in placentome and foetal membrane. Specific antibody was not detected in the serum of this foetus. Foetus #97 was examined at 94 days, 14 days after inoculation. Viable virus was recovered from cerebrum, placentome and spleen, while viral antigen was detected in cerebrum, placentome and spinal cord. This foetus was macroscopically normal, and only mild microscopic lesions were seen in cerebrum, cerebellum, meninges, skeletal muscle and foetal membrane. Specific serum antibody was detected. Foetus #92, examined at 103 days after 14 days' exposure, was dead when recovered. Virus was recovered from placentome, but no other tissues were examined. Foetus #77 was also examined at 103 days, 14 days after inoculation. Virus was re-

covered only from placentome but was unsuccessfully tested for in the other tissues, although viral antigen was detected in cerebrum. The only morphological abnormality detected was mild necrosis and infiltration of the cerebrum. Specific antibody was detected in the serum. Foetus #104 was examined at 114 days, 14 days after inoculation. Virus was recovered only from placentome, while antigen was detected in a wide range of tissues, namely cerebrum, spinal cord, placentome, skeletal muscle and foetal membrane. The only morphological changes were microscopic and consisted of mild lesions in cerebrum, meninges, placentome and foetal membrane. The specific antibody level in the serum was high. Foetus #105 was examined at 134 days, 14 days after inoculation. Virus was recovered only from the placentome, while antigen was detected in cerebrum. No other tissues were assayed for viral antigen. Only microscopic lesions were observed, and these were mild characteristic lesions of cerebrum, spinal cord, meninges, placentome and foetal membrane. In addition to the usual mononuclear cells, neutrophils were present in the cerebral infiltrate. The serum level of specific antibody was high.

Examination of the foetal tissues for the presence of viable virus revealed that this was invariably present in placentomes (Tables 6.1.1 and 6.1.2). However, virus could not be isolated from any of the other tissues of foetuses older than 100 days at autopsy, despite the fact that a substantial number of tissues from these foetuses was examined. All of the foetuses which were examined later than 100 days had been infected 14 days previously. Virus was isolated from brain and spleen of the 94-day foetus, but the titres obtained from these tissues were extremely low. This

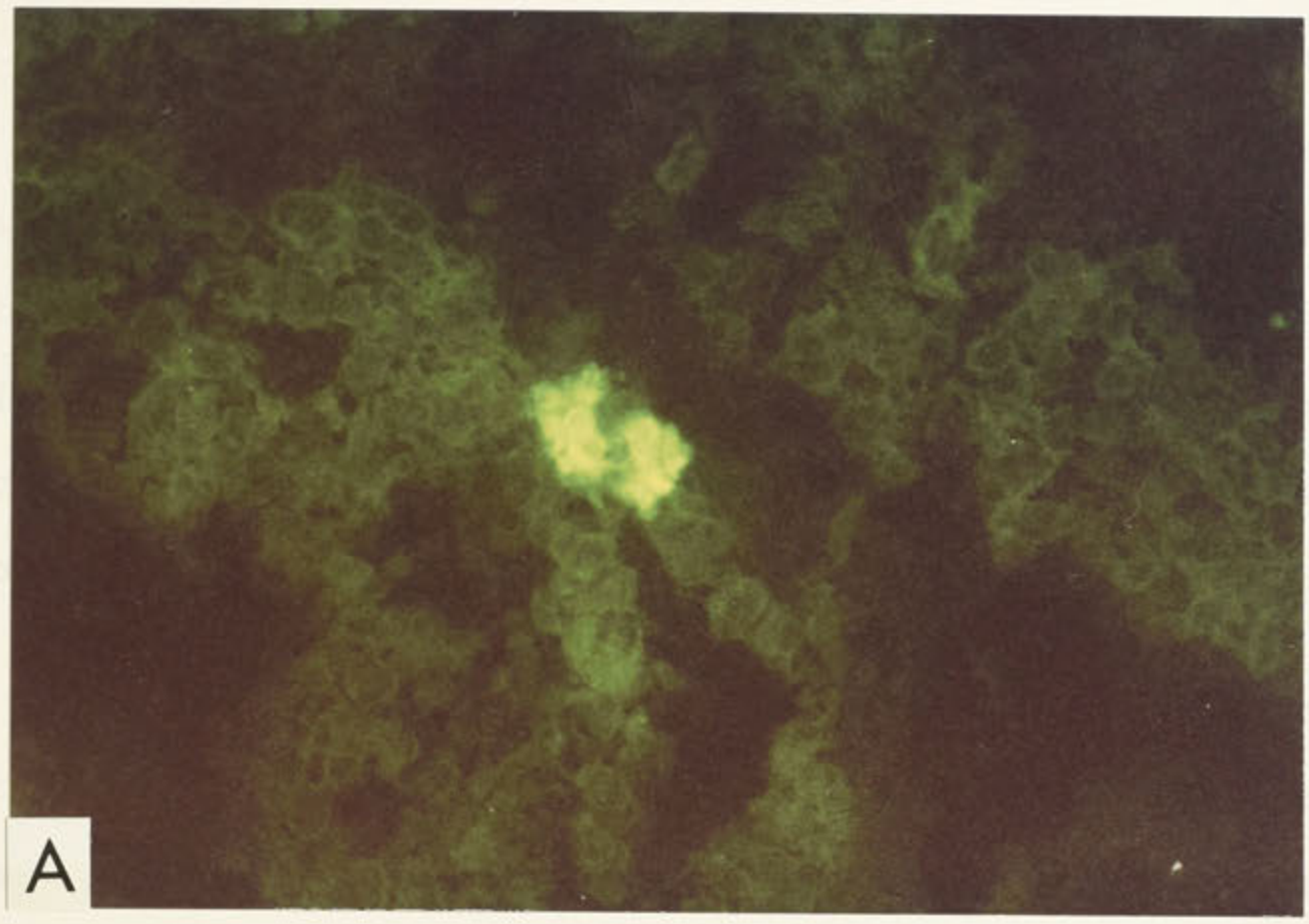
foetus also had been infected 14 days previously. All foetuses of 71 days gestation or younger had high viral titres in brain, foetal membranes and fluids, and skeletal muscle, while virus was rarely if ever recovered from other tissues. These foetuses had been infected five to 15 days previously.

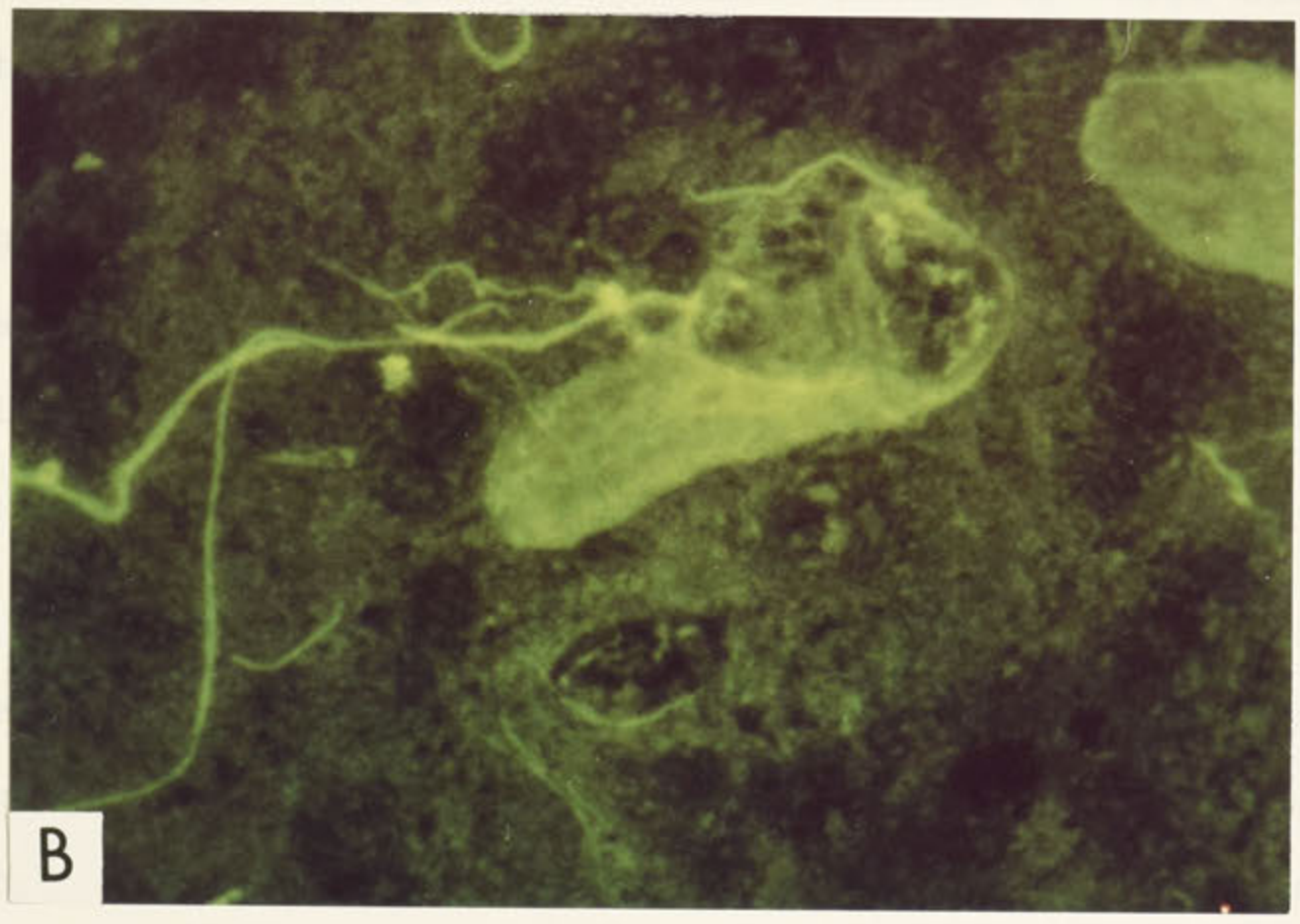
In general, immunofluorescent staining revealed that viral antigens were present in the same range of tissues as live virus (Table 6.2). Plate 6.1 However, individual instances in which a particular tissue was positive by one test and negative by the other were not uncommon. This discrepancy was particularly noticeable in foetuses older than 100 days at autopsy. In these animals antigen was detectable in a range of tissues despite the identification of viable virus only in placentomes. However, in the younger foetuses, tissues were more often positive by plaque forming assay (assaying live virus) than by fluorescent labelling of viral antigen. One would expect the quantity of viral antigen present in infected tissues to be at least equal to the quantity of viable virus irrespective of the test used. This suggests that the plaque assay was a more sensitive system for the detection of Akabane virus than was the fluorescein labelling. If this were so, the presence of viral antigen in tissues from which viable virus was not recovered would indicate that it was inactivated virus which was being detected. As already observed with foetuses infected as a result of inoculation of the ewe, the central lymphoid tissue (liver, spleen and thymus) rarely ever stained positively for viral antigen. When antigen was detected in the liver, it was confined to a few scattered cells similar in type to those affected in transplacentally

PLATE 6.1 Fluorescein-labelled Akabane antigen in tissues of intraperitoneally infected foetuses.

A: # 24, aged 70d, skeletal muscle.
Specific fluorescein labelling occurred only in isolated groups of muscle fibres.
x 250.

B: # 77, aged 103d, cerebrum.
Specific labelling of neuronal cytoplasm and cell processes, is visible.
x 1000.


A


B

infected foetuses (Chapter 5).

The pathological lesions observed in infected foetuses are summarized in Table 6.3. As gross or microscopic abnormalities were not observed in any control foetus, these results are not tabulated. The three young foetuses infected by I/V injection, and the two older I/P-infected foetuses which died were in an advanced state of decomposition when recovered, and no examination for lesions was possible.

Gross lesions were present in five of the remaining 12 foetuses, all infected between day 50 and day 80, and recovered as early as five days later. Macroscopic lesions were not observed in foetuses infected at or after 89 days although, unlike the younger group of foetuses, none of these older foetuses were autopsied earlier than 14 days after infection. The morbid anatomy observed was similar throughout the age range involved, and consisted of increased volumes of peritoneal and pericardial fluid, micrencephaly, fluid-filled hollow cerebral hemispheres, injected and oedematous meninges, and decreased bulk of hindquarter muscles.

Microscopic lesions were present in all 12 foetuses (aged from day 58 to day 134). While similar abnormalities occurred throughout the age range, these appeared to be more prominent in foetuses inoculated in the 50 to 80 day period than in those undertaken at or after day 89, the younger subjects showing more abnormalities, and with a greater degree of severity. These lesions chiefly involved central nervous tissues (especially cerebrum), meninges and skeletal muscle. Hyperaemia and mononuclear cell infiltration of meninges were

observed, the meninges frequently becoming grossly thickened and indistinguishable from the underlying cortex (Plate 6.4, D). Brain lesions (both cerebrum and mid-brain) consisted of focal necrosis, malacia, cavitation, focal haemorrhage, perivascular cuffing, mononuclear cell infiltration which appeared to be focal initially but later became generalized, and in severe cases, total loss of parenchymal architecture (Plates 6.2, 6.3 and 6.4). The infiltrating cells were consistently mononuclear (lymphocytes and some monocytes) except in the case of #104, where the infiltrate also contained polymorphs (neutrophils) (Plate 6.4, B). Severe meningeal infiltration appeared to precede the onset of infiltration into the cerebrum. The extent of cerebral infiltration could not be correlated simply with the maturity of these lambs, as it was less marked in the older foetuses in this group. The severity of infiltration did, however, correlate closely with the period elapsed between inoculation and necropsy. Infiltration was mild if the foetus was examined five days after inoculation but became marked if the interval was extended to 14 days. Focal necrosis and mild lymphocyte infiltration were seen in spinal cord of half of the foetuses. Cerebellar pathological changes were observed in foetuses of 70 to 94 days gestation, and consisted of necrosis of Purkinje cells (a similar lesion is illustrated in Plate 7.1), and a decrease in the number of cells in the granular layer.

Abnormalities of thymus or liver were not seen in any foetus. Three of the five spleens examined showed increased development of white pulp (especially in thymus-dependent areas). Three of five thyroids contained decreased amounts

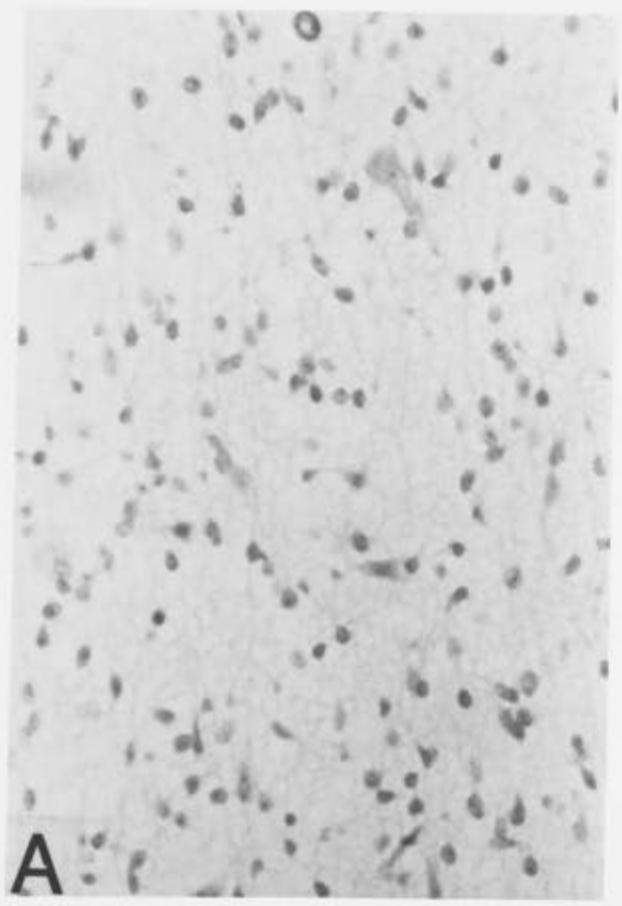
PLATE 6.2 Microscopical lesions of foetal cerebrum after infection with Akabane virus.

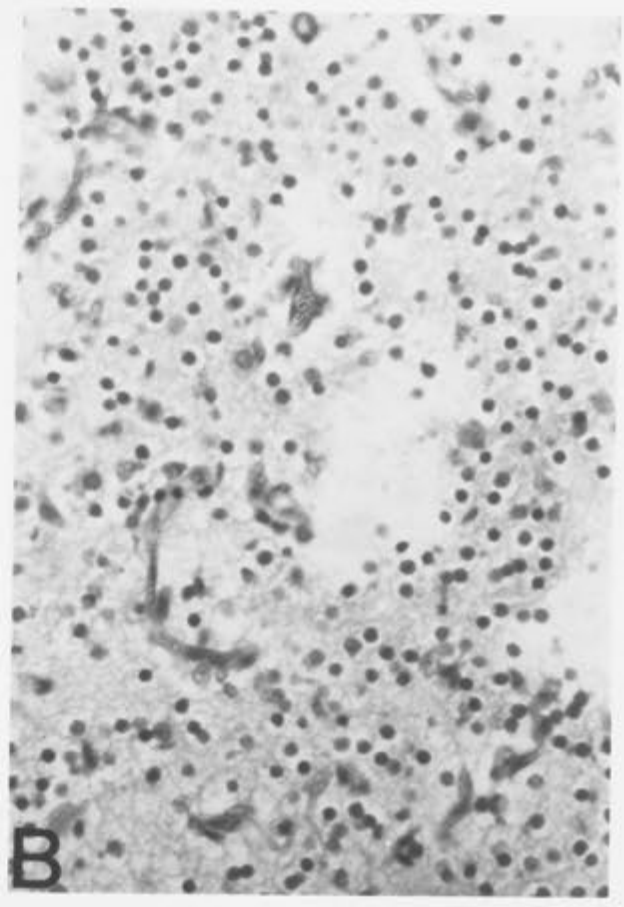
A: Normal appearance of the intermediate zone of the cerebrum from a foetus aged 144d, 14d after subcutaneous inoculation with Vero antigen. Haematoxylin and eosin, x 250.

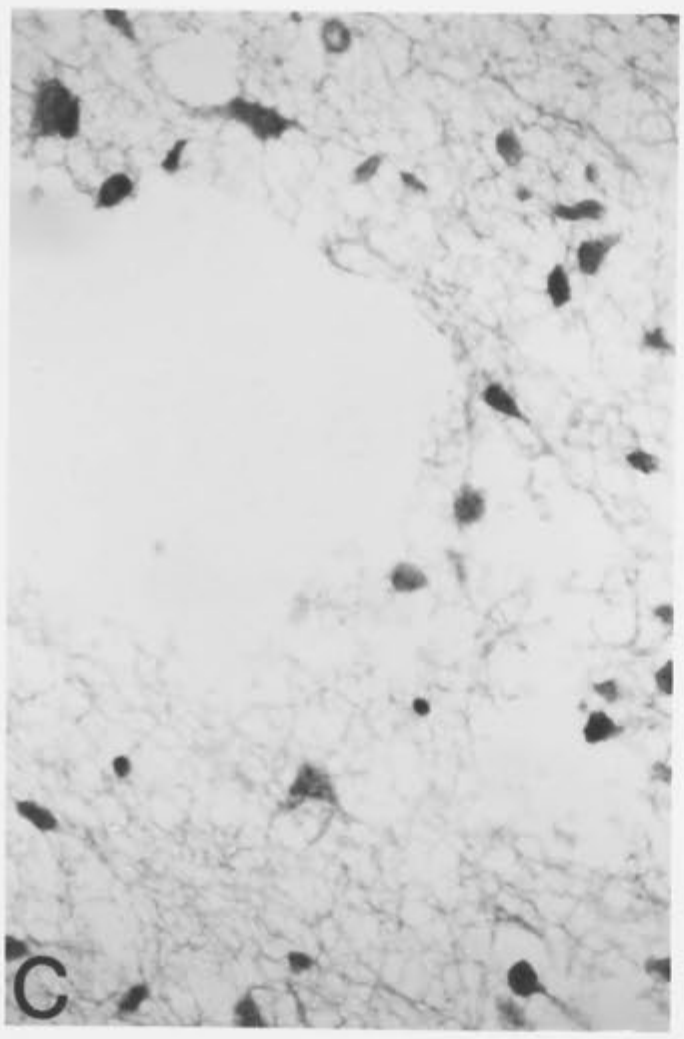
B: Focal necrosis and cavitation of the intermediate zone of the cerebrum, from a foetus aged 89d, 9d after intraperitoneal inoculation with Akabane virus. The region is diffusely infiltrated by mononuclear cells.
Haematoxylin and eosin, x 250.

C: Focal necrosis and cavitation of the intermediate zone of the cerebrum from a foetus aged 58d, 8d after infection with Akabane virus. All cells within the focus have been destroyed. A few infiltrating lymphocytes are present in surrounding parenchyma.
Haematoxylin and eosin, x 400.

D: Focal necrosis and cavitation of the intermediate zone of the cerebrum from a foetus aged 75d, whose mother was infected at 31d with Akabane virus. No cellular infiltration has occurred.
Haematoxylin and eosin, x 400.


A


B


C

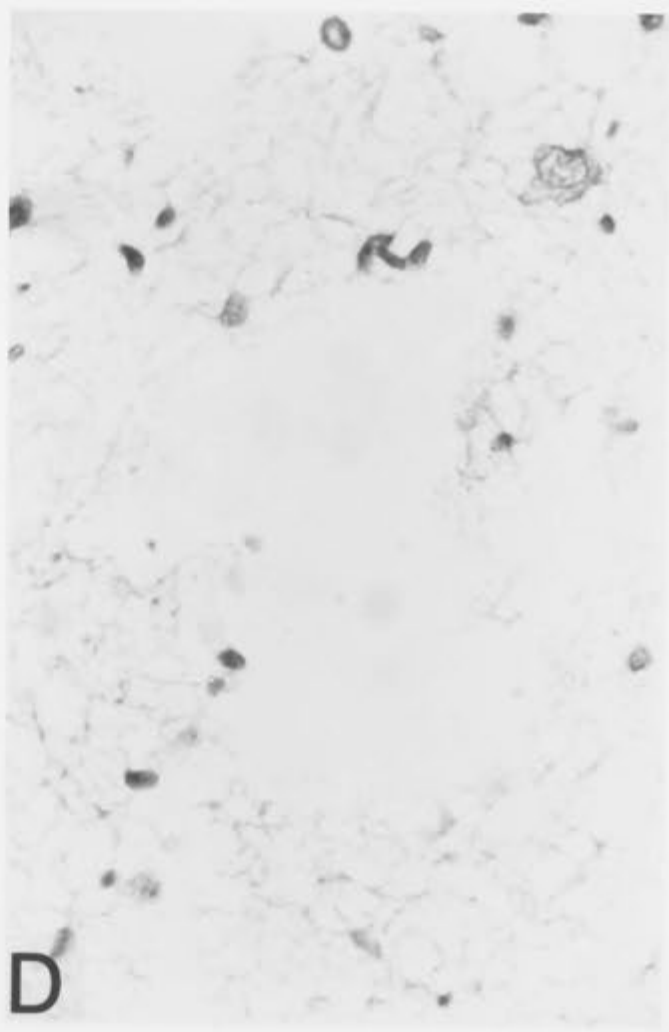
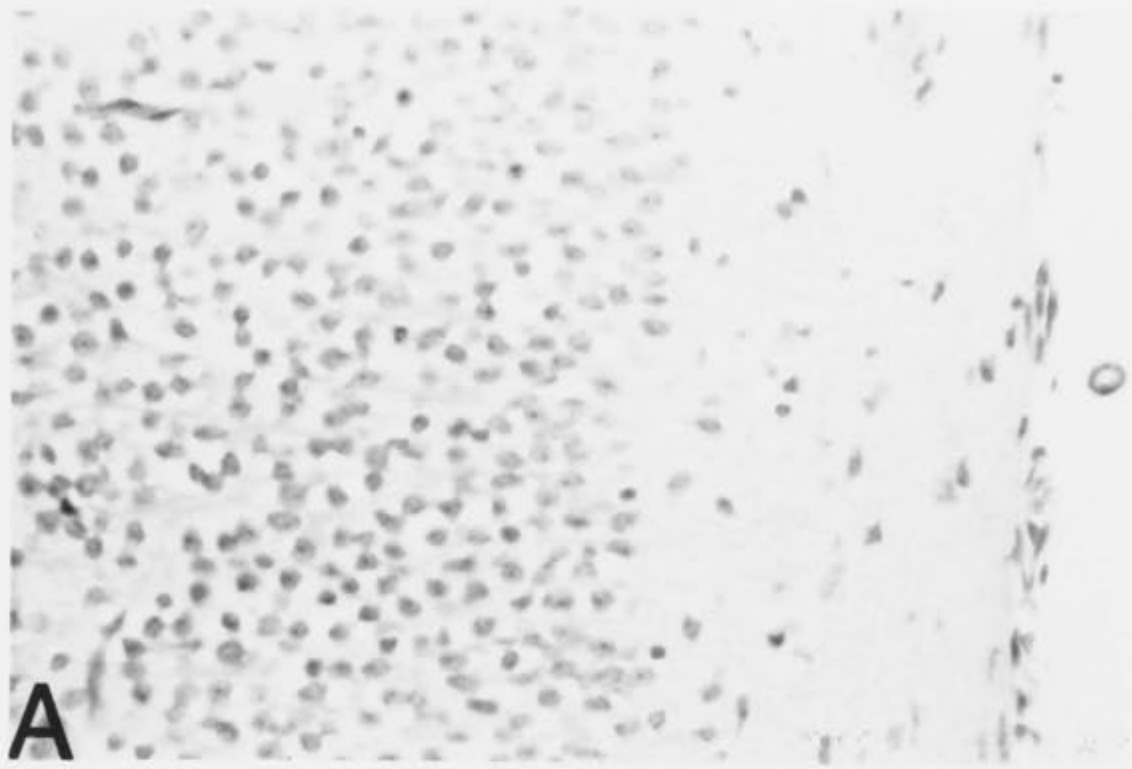

D

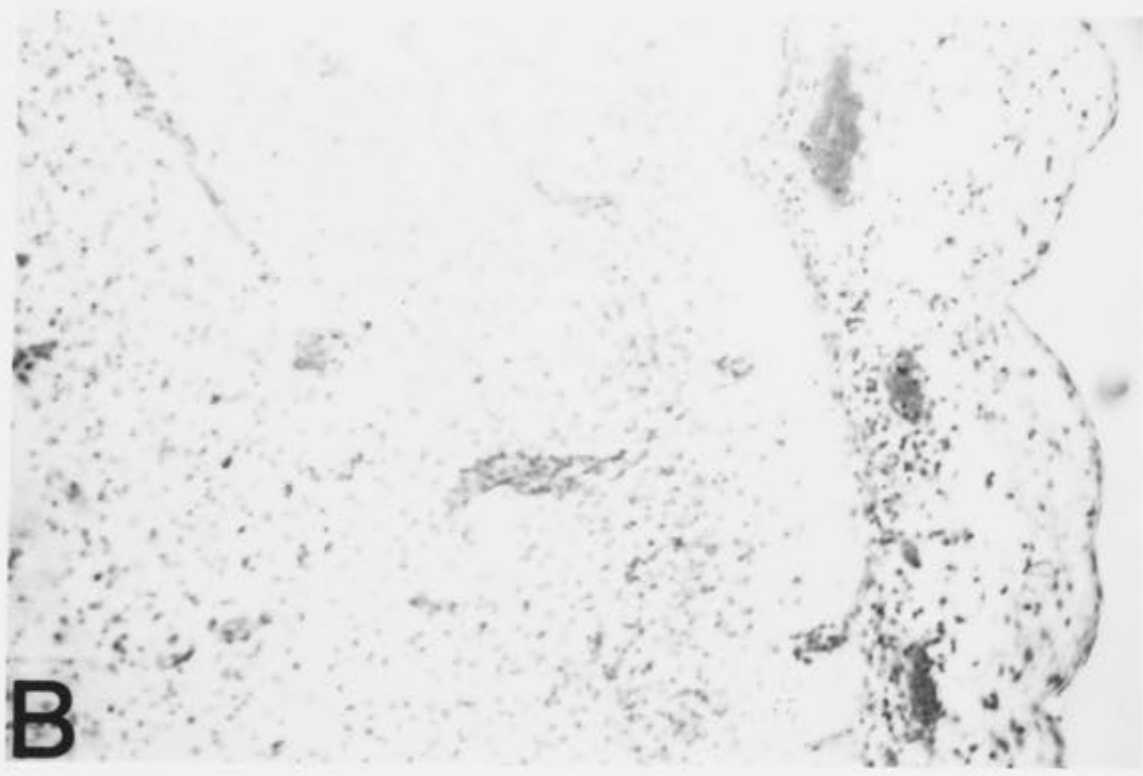
PLATE 6.3 Microscopical lesions of the foetal cerebrum after infection with Akabane virus, continued.

A: Normal cerebral cortex of foetus aged 144d, with meninges at right.
Haematoxylin and eosin, x 250.

B: Cerebral cortex of foetus aged 89d, 9d after infection with Akabane virus. The meninges (at right) are grossly thickened and infiltrated by mononuclear cells (chiefly lymphocytes). Perivascular cuffing is visible in the cortex.
Haematoxylin and eosin, x 100.

C: Cerebrum of foetus aged 134d, 14d after infection with Akabane virus, showing perivascular cuffing by mononuclear cells, chiefly lymphocytes.
Haematoxylin and eosin, x 250.


A


B


C

PLATE 6.4 Microscopical lesions of foetal cerebrum after infection with Akabane virus, continued.

A: Intermediate zone of cerebrum of a foetus aged 122d, 14d after subcutaneous infection with Akabane virus, showing a focus of mononuclear cell infiltration. Infiltrating cells were chiefly lymphocytes, but some monocytes were present.
Haematoxylin and eosin, x 400.

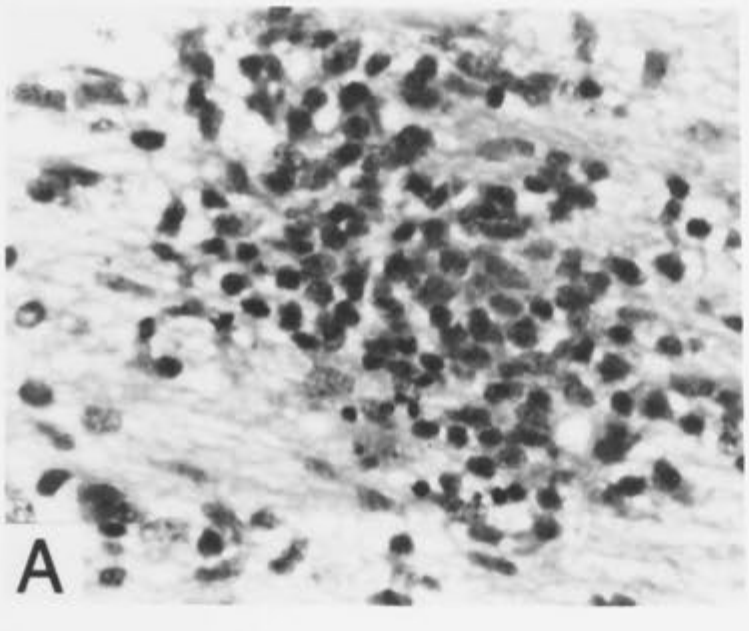
B: Intermediate zone of cerebrum of a foetus aged 134d, 14d after intraperitoneal infection with Akabane virus. In this foetus, the focal cellular infiltrate was composed of both mononuclear cells and polymorphs - chiefly lymphocytes and neutrophils. Some monocytes were present.
Haematoxylin and eosin, x 400.

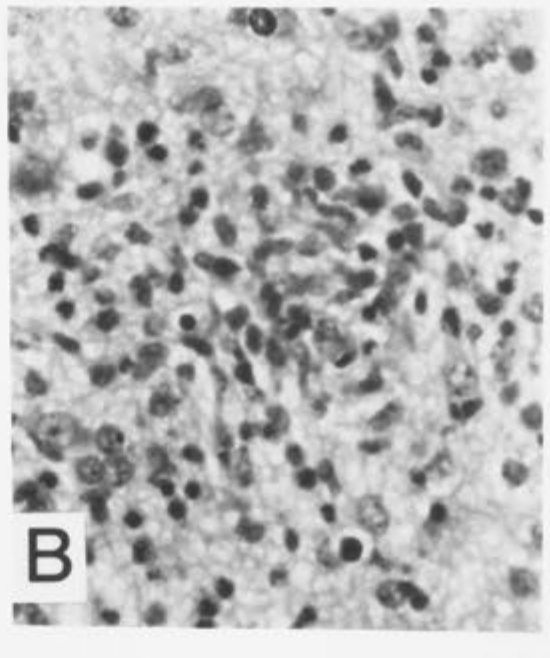
C: Primitive cerebral cortex of a foetus aged 89d, 9d after intraperitoneal infection with Akabane virus. The tissue architecture is being destroyed, but the meninges (top) are still identifiable. The cellular infiltrate is generalized and consists of lymphocytes and monocytes. There is extensive necrosis of parenchymal cells, and macrophages are visible.
Haematoxylin and eosin, x 250.

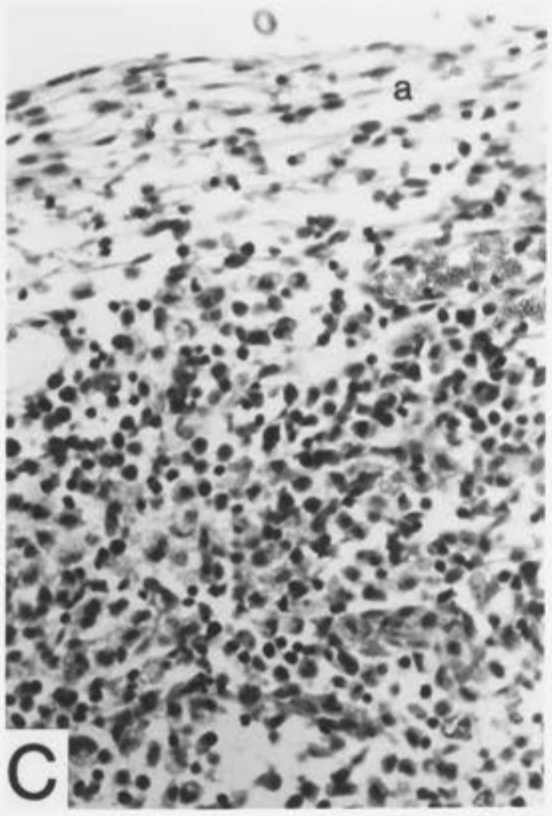
D: Another region of the section shown in C. The tissue architecture has been completely lost, and the meninges (again at top of frame) are not identifiable.
Haematoxylin and eosin, x 250.

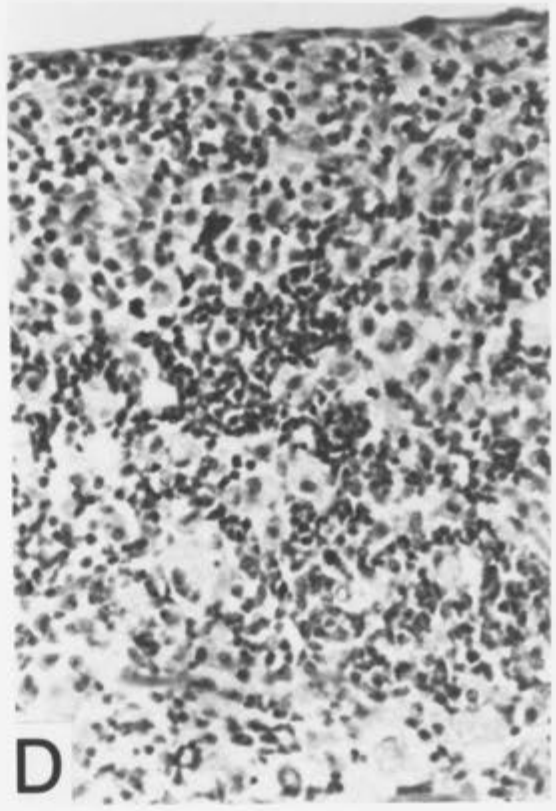
E: Primitive cerebral cortex of a foetus aged 75d, 15d after intraperitoneal infection with Akabane virus. Large numbers of lymphocytes and active macrophages are present, and there is extensive necrosis of brain parenchymal cells and of immigrant cells.
Haematoxylin and eosin, x 400.

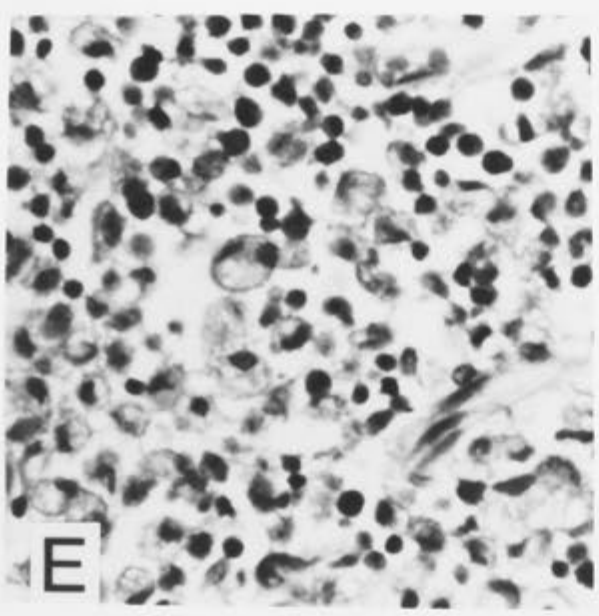
F: Primitive cerebral cortex of a foetus aged 70d, 15d after intraperitoneal infection with Akabane virus. This lesion is a more severe form of that shown in E; most of the cells are necrotic and only cell debris remains.
Haematoxylin and eosin, x 400.

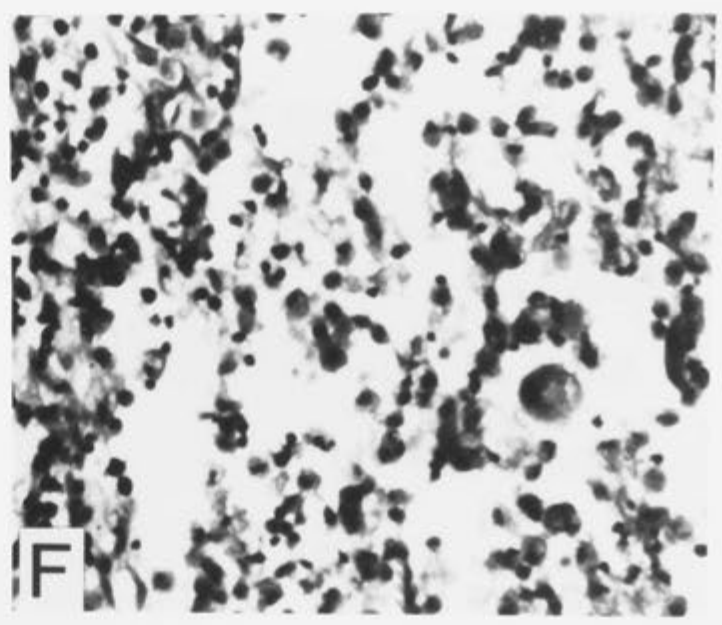

A


B


a
C


D


E


F

of colloid.

Focal haemorrhage occasionally occurred in lung and kidney, as did focal mononuclear cell infiltration. One foetus (#24) had renal tubular necrosis and replacement by fibrous tissue. Focal mononuclear cell infiltrate was observed occasionally in cardiac muscle.

Placental damage had occurred consistently, in contrast with the rare cytotoxicity of the virus for the placenta of the foetuses described in Chapter Five. These placental lesions although common were usually mild, and consisted of focal necrosis of placental trophoblast and/or syncytium, often involving only a few cells (Plate 6.5, C and D). Sometimes they were accompanied by oedema and perivascular cuffing in foetal membranes (Plate 6.5, B). The trophoblast was often thickened at sites of necrosis and these regions also stained positively for viral antigen. In only one foetus (#24, 70 days) was there total necrosis of some foetal villi (Plate 6.5, E).

The principal skeletal muscle lesion was focal necrosis of fibres (Plate 6.6). This frequently left the sarcolemma intact and so gave the appearance of adipose tissue replacement. Replacement of muscle fibres by connective tissue was only rarely observed (in #23 and #35) and was restricted to foci. Focal haemorrhage and perivascular cuffing also occurred in skeletal muscle, as did focal infiltration by lymphocytes and monocytes (Plate 6.6, E).

The serum neutralizing antibody titres for foetuses and ewes are shown in Table 6.4. While the adults seroconverted

PLATE 6.5 Microscopical lesions of the foetal placenta after infection with Akabane virus.

A: Normal placentome at 75d gestation, with foetal trophoblast top right.
Haematoxylin and eosin, x 400.

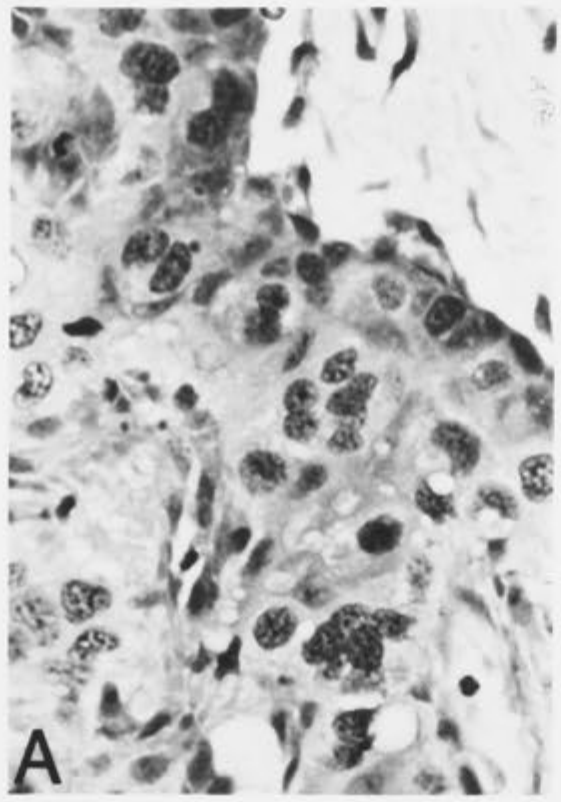
B: Placentome at 70d gestation, from foetus infected at 55d gestation with Akabane virus. The foetal membrane shows perivascular cuffing by monocytes.
Haematoxylin and eosin, x 250.

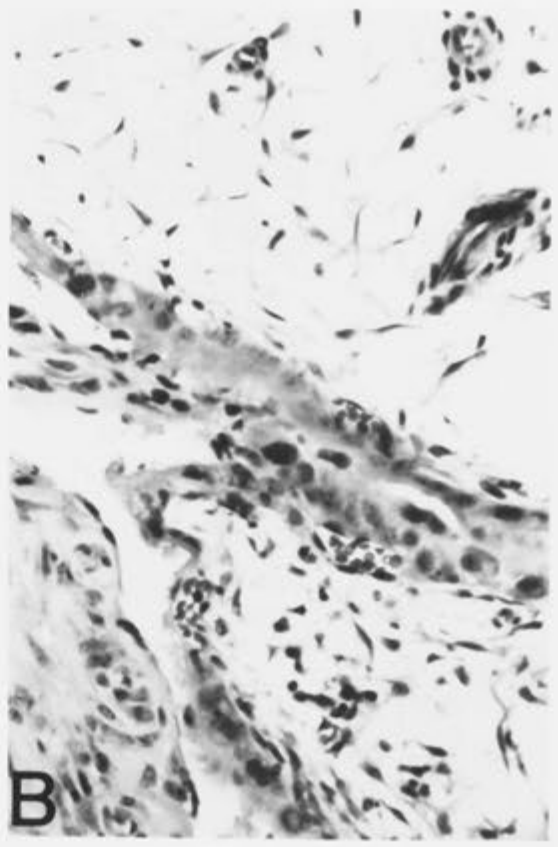
C: Placentome at 70d gestation, from foetus infected at 55d with Akabane virus. The foetal trophoblast (top) has a focus of cell necrosis and trophoblast thickening.
Haematoxylin and eosin, x 400.

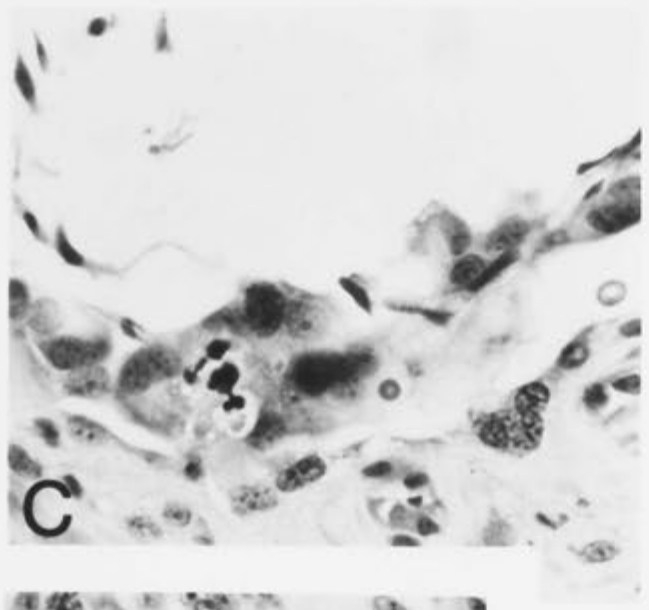
D: Placentome at 75d gestation, from foetus infected at 60d with Akabane virus. There is focal necrosis of cells in both trophoblast (right) and syncytium.
Haematoxylin and eosin, x 400.

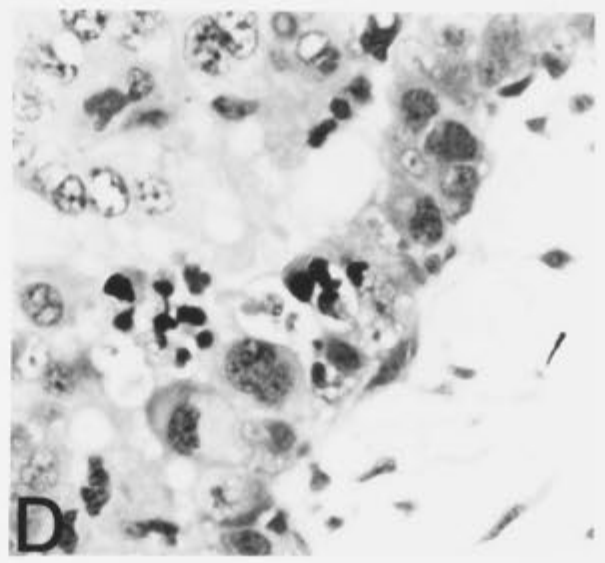
E: Placentome at 70d gestation, from foetus infected at 55d with Akabane virus. The foetal villus is totally necrotic, while the maternal tissues appear normal.
Haematoxylin and eosin, x 400.

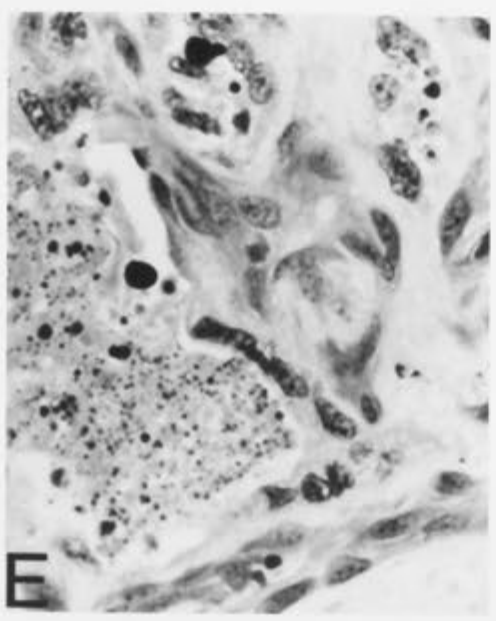
F: Placentome at 122d gestation, from foetus infected at 109d with Akabane virus. Both foetal and maternal tissue are necrotic, and there has been extensive infiltration by maternal leucocytes.
Haematoxylin and eosin, x 400.


A


B


C


D


E

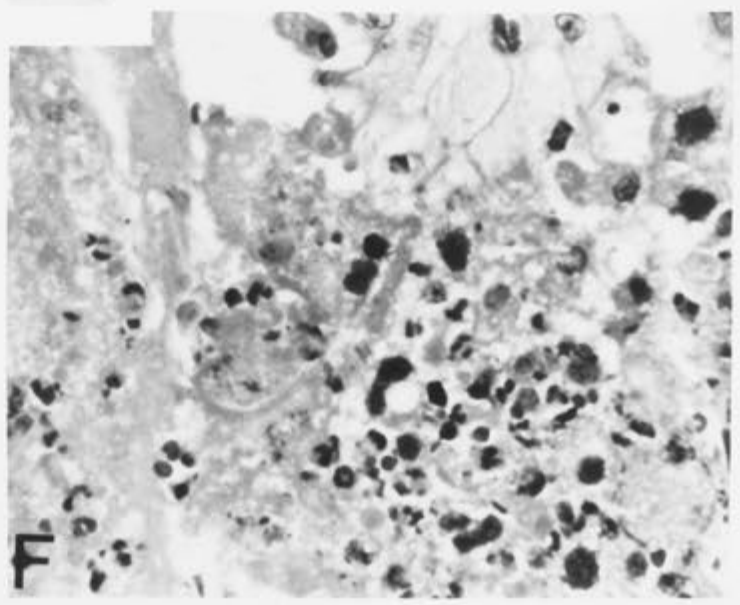

F

PLATE 6.6 Microscopical lesions of foetal skeletal muscle after infection with Akabane virus.

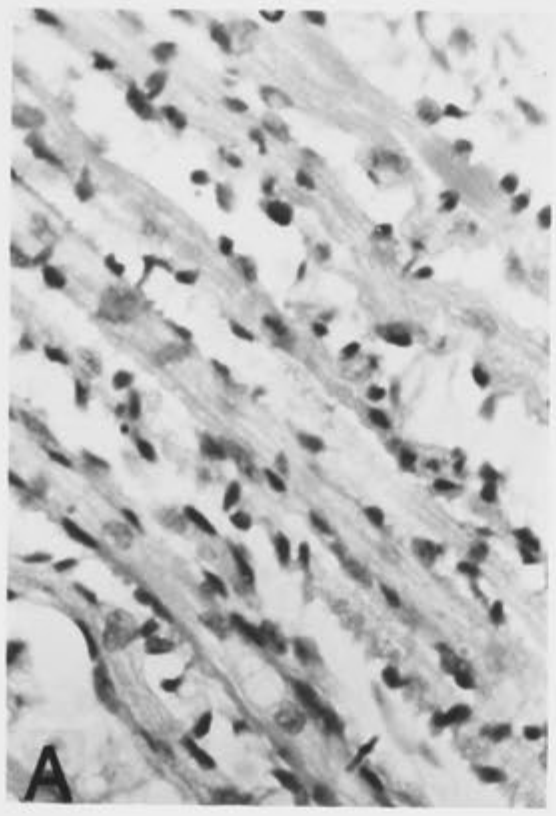
A: Normal skeletal muscle from a control foetus aged 76d. Longitudinal section. Haematoxylin and eosin, x 400.

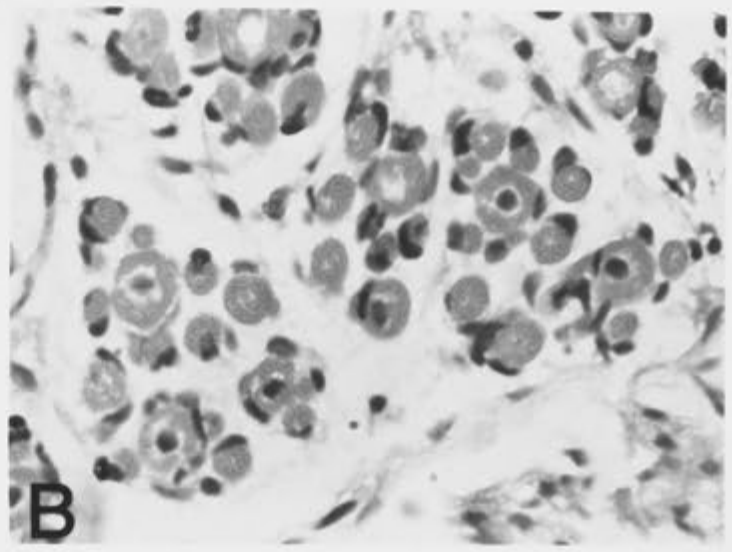
B: Normal skeletal muscle from a control foetus aged 76d. Transverse section. Haematoxylin and eosin, x 400.

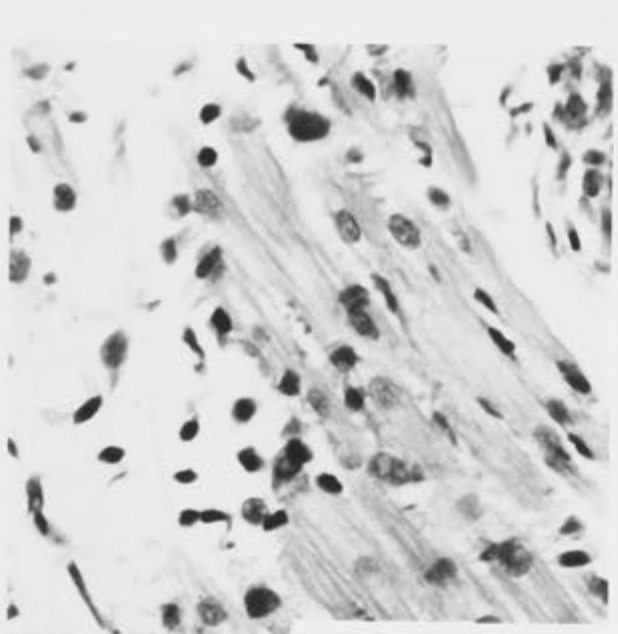
C: Skeletal muscle from a foetus aged 75d, 15d after infection with Akabane virus. There has been focal necrosis of fibres leaving cell debris and pyknotic nuclei. Haematoxylin and eosin, x 400.

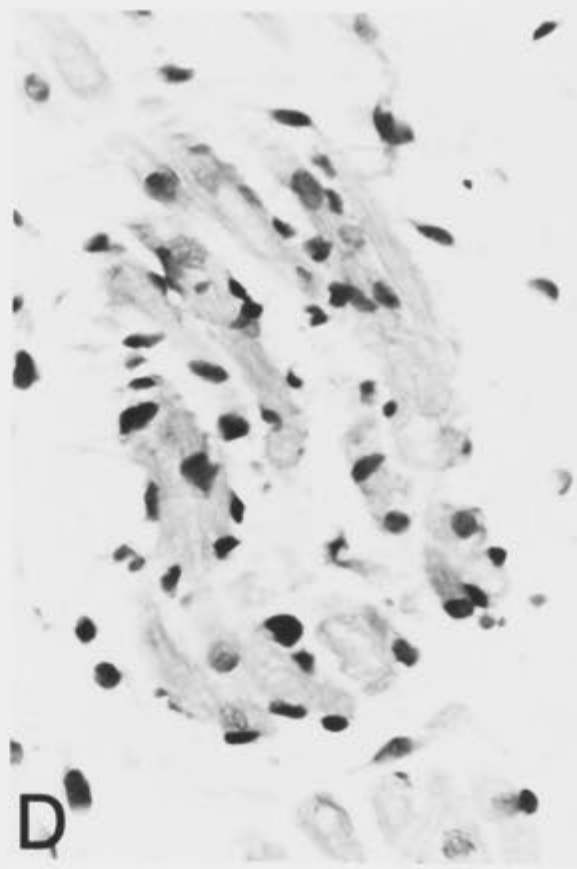
D: Skeletal muscle from a foetus aged 75d, 15d after infection with Akabane virus. Focal necrosis of myocytes has occurred in this group of fibres. Haematoxylin and eosin, x 400.

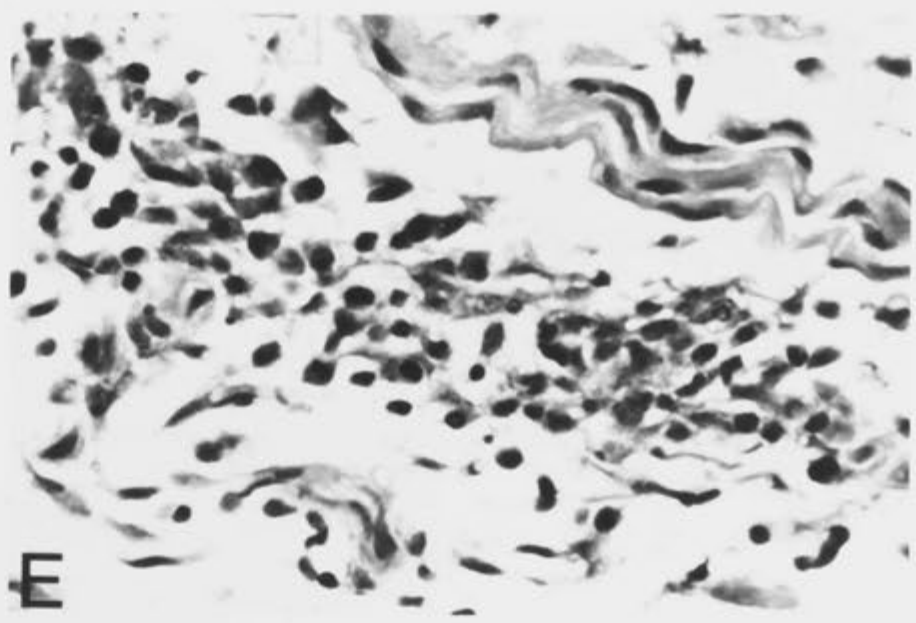
E: Skeletal muscle from a foetus aged 75d, 15d after infection with Akabane virus. Focal necrosis of a group of muscle fibres is accompanied by infiltration by lymphocytes and monocytes. Haematoxylin and eosin, x 400.


A


B


C


D


E

by the time the foetus was removed (5-15 days after infection), specific antibody was only detected in sera from foetuses of 75 days or older, which had been inoculated 15 days earlier. Even when foetal serum contained detectable levels of specific antibody, foetal allantoic fluid did so only once, in a foetus inoculated at 80 days and sampled 14 days later. The in vitro responses of foetal cells to mitogens and to Akabane virus are shown in Table 6.5. It is likely that the single time after infection at which these cells were assayed did not coincide with the time of peak responsiveness of the foetal cells. Spleen cells from two of the five lambs examined responded to incubation with Akabane virus by proliferation significantly above that produced by incubation with Vero control alone. This increase in stimulation index was apparent in foetuses sampled 14 days after they were inoculated at 80 and 89 days (spleen cells from foetuses inoculated earlier than 80 days were not tested), and was not evident in the only control foetus examined (75 days, see Table 5.6, Chapter 5). The S.I. of these positive foetuses were similar to those resulting from Akabane viral stimulation of liver cells collected from the four reactive foetuses from inoculated mothers (see Chapter 5), but lower than those observed for lymphocytes from immunized adults. Spleen cells from foetuses of 103 days and older also responded well to Con A, while spleen cells of 94 days responded slightly, and spleen cells of 89 days did not respond. This contrasted with normal spleen cells of 75 days, which responded well to Con A (see Table 5.6, Chapter 5). These S.I. were higher than those resulting from Con A stimulation of liver cells collected from

TABLE 6.5 - Response (^{3}H-thymidine incorporation) of cells from foetuses directly infected by intraperitoneal injection of Akabane virus to mitogen and antigen.

Tissue	Foetus	Age(d) at inoculation	Age(d) at recovery	Controls		Non-specific stimulation		Specific stimulation			
				Medium Cpm	Vero Cpm	Con A Cpm	SI	Akabane Cpm	SI	Inactive Akabane Cpm	SI
Liver	24	55	70	9323	3504	3401	0.4	6583	1.9	5239	1.5
	81	80	89	3394	3127	2277	0.7	574	0.2	1473	0.5
	97	80	94	1048	857	959	0.9	367	0.4	486	0.6
	77	89	103	1013	385	688	0.7	213	0.6	253	0.7
	104	100	114	461	324	183	0.4	216	0.7	195	0.6
	105	120	134	189	132	331	1.8	128	1.0	161	1.2
Spleen	81	80	89	22977	24853	44768	1.9	38354	1.5	24853	1.0
	97	80	94	25267	18813	58188	2.3	39424	2.1	17793	1.0
	77	89	103	12063	13935	148379	12.3	30114	2.2	16693	1.2
	104	100	114	11312	14862	148183	13.1	18875	1.3	14094	1.0
	105	120	134	7888	21004	157174	19.9	38873	1.9	14667	0.7
Thymus	81	80	89	3732	5727	74130	19.9	11410	2.0	3301	0.6
	97	80	94	8818	5238	89495	10.1	10064	1.9	6402	1.2
	77	89	103	1142	546	11638	10.2	854	1.6	580	1.1
	104	100	114	737	607	2906	3.9	290	0.5	254	0.4
	105	120	134	1814	1030	2259	1.2	365	0.4	293	0.3

Cpm = counts per minute of β emission from incorporated thymidine. SI = stimulation index.

SI for Con A = $\frac{\text{Cpm of cultures with Con A}}{\text{Cpm of cultures with medium only}}$ SI for virus = $\frac{\text{Cpm of cultures with virus}}{\text{Cpm of cultures with Vero}}$

the two reactive foetuses of inoculated mothers. They were similar to the S.I. resulting from Con A stimulation of adult lymphocytes collected at a similar interval (14 days) after immunization. Thymus cells from foetuses at 89-103 days gave responses to Con A similar to those of normal foetuses of 71 to 76 days, while thymus cells from infected foetuses of 114 days or older did not so respond (Table 6.5). Only one infected foetus (89 days) showed a thymus cell response to Akabane virus. There was no indication of any responsiveness to Akabane virus or to Con A by liver cells from the same foetuses. However, the ages at the time of assay (80 days and older) were such that the lymphoid cell content of the liver was likely to have been very low. Non-specific competence of thymus and spleen cells was invariably present by 71-75 days in normal foetuses (Table 5.6). However, in five infected animals, while thymic competence was demonstrated by 89 days (which was the earliest age at which they were tested), splenic lymphocytes appeared not to become non-specifically competent until between 94 and 103 days.

6.4 DISCUSSION

In vitro lymphocyte transformation assays were performed for each foetus at a single time after infection. In view of the results in Chapter Four, in which the response to mitogens and to Akabane virus varied with the interval after infection, it is probable that peak responses of foetal cells would frequently not be revealed by single point assays. Thus the variation observed in the cellular responses could have several possible explanations including variation in the post-infection interval, variation in the stage of maturation of

different foetuses and, perhaps, the occurrence of virus-induced immunosuppression.

Comparison of the response to Con A of spleen cells from infected and control foetuses suggests that Akabane infection may have delayed attainment of the capacity to mount a non-specific response from the anticipated 75 days to later than 94 days. This suppression was associated with the presence of viable virus in the foetus at the time of testing (see Table 6.1) as it occurred in foetuses from which viable virus was widely distributed, but not in those from which virus was found only in placentomes. This tends to confirm the suggestion made in Chapter Two that Akabane virus infection may depress the in vitro response of liver cells to Con A, and agrees with the failure to observe cellular or humoral response to infection described in Chapter Five. However it contrasts with the results of in vitro stimulation of normal adult lymphocytes by Con A plus virus (Chapter 3) which showed no viral influence on in vitro response to Con A. Depression may require in vivo challenge, or may be temporary only, as cells from Akabane challenged adults showed a transitory decrease in response to Con A stimulation before the response increased (Fig. 3.2). Alternatively, Akabane virus may affect only foetal immunocompetent cells, while adult lymphocytes are insusceptible to its suppressive action. The response of spleen cells from the three other foetuses tested to Con A showed no depression in late gestation, so any viral-induced suppression would appear to be temporary.

The failure of thymus cells from infected foetuses to

respond to specific or non-specific stimuli in late gestation, despite a capacity to respond at earlier ages, also raises the question of immune suppression by suggesting that the thymic lymphocyte response was suppressed before birth. This contrasts with the observation by Leino (1978) of good responses to Con A stimulation by normal foetal thymocytes at 107, 122 and 135 days. Insufficient animals were involved in this study to determine whether previous Akabane challenge was responsible for this discrepancy.

Direct injection of Akabane virus into foetuses of 50-120 days regularly resulted in infection and disease. If, as suggested by Parsonson et al, (1977) and Hashiguchi et al, (1979), there is an age limit beyond which maternal infection does not result in foetal disease, this is unlikely to be entirely explicable in terms of foetal tissue insusceptibility or foetal immune competence. Even those foetuses with detectable humoral or cellular responses to the virus, which were presumed to have affectively inactivated or eliminated the virus, retained demonstrable viral antigen in a wide range of tissues. This was accompanied by histological lesions in central nervous system tissue. Apparently, the virus had infected and damaged cells in these foetuses before an effective immune response could be mounted. Virally induced damage was less severe in foetuses in which an immune response was detected. The humoral component of the anti-virus response appears to have developed by approximately 75 days. Although specific cellular recognition of Akabane virus was not demonstrated until 94 days, an earlier onset could not be excluded because while the histological cellular response observed in

younger foetuses may have been specific, the *in vitro* assay was not performed in foetuses younger than 89 days.

The appearance of specific neutralizing antibody in the serum coincided with the disappearance of viable Akabane virus from foetal tissues other than placentome. There was no consistent correlation between presence of viable virus and viral antigen in individual foetuses although, overall, both were found in the same range of tissues. Viable virus and antigen were widely distributed in tissues from foetuses of 71 days and less, regardless of the interval between inoculation and examination and none of these foetuses had detectable specific antibody. The foetus examined at 89 days gave similar results after only nine days' exposure to the virus. The 94-day foetus yielded low viral titres from a small number of tissues although antigen was still detectable, and was seropositive for specific antibody when examined 14 days after inoculation. When examined 14 days after inoculation, the remaining four foetuses were aged 75, 103, 114 and 134 days. All yielded virus from the placenta only, although viral antigen was detectable in other tissues. All three possessed specific neutralizing antibody. That is, the lack of virus was regularly associated with the occurrence of a humoral immune response, providing the foetus was 75 days or older when tested. Consequently, elimination of virus is likely to have been attributable to an effective foetal immune response. It appears from this study and from that to be described in Chapter Seven that specific antibody appears approximately eight to 11 days after foetal infection. It therefore seems likely that provided a foetus is at least 75 days old and has

had a minimum of approximately ten days' exposure to Akabane virus, it would be capable of mounting a specific humoral response capable of ensuring the inactivation of the virus. Such antibody did not appear to eliminate intracellular viral antigen, nor to inactivate virus present in the placentome. If the antibody was directly responsible for viral inactivation in other foetal tissues, it is curious that it did not do so in trophoblast and/or syncytium. This failure of the humoral response to eliminate virus from the placentome confirms the observation of Parsonson et al, (1981a), and suggests that foetal trophoblast is not accessible to either foetal or maternal immune mechanisms. In addition to the effect of the humoral response, there also appears to be a further influence on viral titres in brain. As seen in Table 6.1.1, the highest titres were found in foetuses which had had the shortest interval between infection and examination, suggesting that even in the absence of specific antibody, virus survives in the brain for only a limited time.

There was also an inverse relationship between the presence of specific neutralizing antibody and the severity of the morphological abnormalities observed. The most likely explanation for this is that antibody-induced inactivation of the virus limited viral necrosis of cells and/or virally induced inflammation.

The pattern of distribution of viral antigen and viable virus did not differ significantly from that observed in younger foetuses infected as a result of placental transmission (Chapter 5). Quantitative assay of the viral content

of tissues revealed that, whereas titres in placentomes were quite similar in transplacentally infected and directly inoculated foetuses, brain titres were much higher in the directly inoculated group after a comparable period of exposure to the virus. This difference ranged from one to five orders of magnitude. The virus is capable of multiplying in placental cells (McPhee, personal communication) but its capacity to multiply in other tissues has not been ascertained. If replication does not occur in brain, the levels of virus attained in this tissue might reflect the virus load to which it is exposed initially, while the levels in the placenta could rise with time to a ceiling irrespective of the infecting dose. While there was a tendency for virus titres in skeletal muscle to be higher in the directly inoculated group of foetuses, these differences were of less than an order of magnitude. Viral titres in the foetal membranes were similar in the two groups of animals.

Histological lesions appeared very soon after direct inoculation (eight days) and no difference in the speed of this appearance at different ages was detected. The severity of the lesions in seronegative foetuses continued to increase with the time after infection. There were no foetal histological lesions eight days after maternal inoculation at 31 days (Chapter 5), but it is not possible to conclude whether this difference is due to the route of infection or the age of the foetuses.

Histological examination of the tissues of directly inoculated foetuses revealed substantial cerebral abnormality

comparable in extent with that previously observed in maternally infected foetuses of similar ages. However, there was a marked difference from the tissues of the indirectly inoculated group in that the brains and meninges of directly inoculated foetuses were regularly characterized by cellular infiltration, whereas the only indirectly infected foetus to show such infiltration was the one from the ewe infected at 44 days. This infiltration may have been an antigenically specific response to the presence of the virus and thus responsible for some of the cytolysis, or it may have been a non-specific reaction to virally induced tissue damage. Since the lack of polymorphs in all but one of the infiltrates may have been due to an overall scarcity of polymorphs at this age rather than because the infiltrate was a specific cytotoxic T-cell response, it is not possible to distinguish between these alternatives. This infiltration was most evident in foetuses infected between day 50 and day 80. Several explanations are possible for this timing. Perhaps such infiltration occurs only in the absence of specific neutralizing antibody. Alternatively, it may occur only after competence is sufficiently developed but before virus has been eliminated. In this case, it would indicate that the capacity for mononuclear cell chemotaxis has developed by 58 days. It is thus interesting that with one exception, foetuses described in Chapter Five, which were transplacentally-infected, did not show any cellular infiltration of the central nervous system. A third possibility could be that it occurs only at a transient stage in the development of immunocompetence, possibly due to deficient control mechanisms.

Since at the time of examination indirectly inoculated foetuses were of comparable age at examination with directly infected foetuses showing severe cellular infiltrate, their lack of infiltrate may be accounted for by a difference in dose received. The total virus dose administered by direct injection into the foetus is likely to be higher than that received after transplacental transmission from the viraemic ewe. A higher infecting dose may result in higher viral titres in target tissues (cf Table 6.1 and Table 5.1) and therefore a greater local stimulus for the immigration of sensitized lymphocytes. The most marked difference in viral titres between the two routes of infection occurred in the central nervous system tissues, and these were also the tissues in which most infiltration occurred following direct foetal infection. Alternatively, the different pattern of exposure may affect the target tissue susceptibility or the foetal defences, immunological or otherwise. Direct inoculation provides a single high dose (pulse) of virus, while transplacental infection probably results in the continued release of a lower level of virus from the reservoir in the placentome. A pulse of virus may penetrate more effectively to target tissues than a continuously released dose, while a continuously released low dose may be limited by a host immune response, or may modify that immune response so that it does not mount the cell-mediated inflammatory response. For example, it may induce tolerance.

Alternatively, the duration of exposure to the virus may influence the inflammatory response, since the directly and indirectly infected foetuses examined at comparable ages

frequently varied in their duration of exposure. The presence of infiltration in the brain of the foetus indirectly infected at 44 days suggests that foetal age at the time of infection might be important. If so, this influence might be exerted by one of the mechanisms suggested above, namely stage of immunocompetence, relative dose and pattern of exposure, or by variation in the tissue susceptibility to virus.

While it is likely that a primary viral cytopathic effect suffices to explain the intracranial deformity, clinical disease may well be magnified by a host inflammatory response once the capacity to mount this has been acquired.

The inflammatory response usually involved only mononuclear cells (lymphocytes and monocytes). The only foetus to show a polymorphonuclear infiltration was also the oldest to show any inflammatory response (134 days at recovery). The reported time for first appearance of circulating polymorphs is 60 days (Sawyer et al, 1978), thus one would expect that by 114 days there would be sufficient polymorphs available for recruitment. In view of the findings of Sawyer et al. (1978), that neutrophils are neither fully differentiated nor fully functional until birth or shortly before, it is most likely that the phenomenon is age-dependent, and that the absence of polymorphs in cellular infiltrations before 134 days of gestation is due to functional incompetence on the part of the polymorphs. Alternatively, Akabane virus may normally only induce a mononuclear cell infiltrate, and #105 may have produced an abnormal response. There is a third possibility, namely that the nature of the cellular infiltrate

was influenced by the time after infection at which the foetus was examined. For example, polymorphs might only be prominent during the first week of the response. This explanation seems unlikely as polymorphs were not detected in infiltrates in foetuses infected at 60 to 80 days and examined five to nine days later, and the only infiltrate with polymorphs was in a foetus examined at 14 days after infection.

The specificity of infiltration in tissues of infected foetuses (severe in central nervous system only, and mild in skeletal muscle) suggests that the virus is present there in the greatest quantity, even in foetuses older than 100 days. The degree of the inflammation seen after 100 days may be milder because no viable virus remains to stimulate a cellular response and/or chemotaxis, or because such a response has been better controlled, or suppressed.

In conclusion, the foetal lamb is susceptible to virus-induced damage when directly infected with Akabane virus between 50 and 120 days. This damage is contributed to both by primary viral-induced necrosis and possibly also by depression of parenchymal cell division in target tissues, and by lymphocytic inflammatory infiltration. Specific humoral response to infection was first observed at 75 days and failure to recover viable virus from tissues was associated with the appearance of antibody. Specific <u>in vitro</u> lymphocyte response was slight and inconsistently observed from 94 days.

CHAPTER 7

THE RESPONSE TO LOCAL CHALLENGE OF THE FOETAL LAMB WITH AKABANE VIRUS

CHAPTER 7 - THE RESPONSE TO LOCAL CHALLENGE OF THE FOETAL LAMB WITH AKABANE VIRUS

7.1 INTRODUCTION

These experiments involved the cannulation of an efferent lymphatic duct of foetuses in late gestation (104 to 126 days) and challenge of the local node with Akabane virus, followed by examination of the behaviour of the virus in the host, its effect on the host, and the response of the host's immunological system. A considerable amount of data on the local lymphoid response to various foreign antigens has been gathered from adult sheep, using essentially the same protocol of cannulation (Lascelles and Morris, 1961; Hall et al, 1962; Pearson et al, 1976; Trnka and Cahill, 1980). The aims were two-fold. Firstly, they were designed to study the local response to the infection. The second aim was to investigate the role of lymphocytes in harbouring and transporting Akabane virus after foetal infection.

The first point addressed by these experiments was the nature of the local foetal response to Akabane virus. Previous work with the foetus was concerned with the systemic response following the systemic route of inoculation, and relied on observations taken at a single time for each foetus. Serial examination of the local lymphoid response to infection by the subcutaneous route was performed to provide more insight into the early events following viral challenge of the foetus. In particular, determination of the time elapsing between inoculation and onset of the different components of the immune response was expected to permit not only the correlation

between humoral and cellular events, but also the correlation of immunological events with the behaviour of the virus and with development or diminution of disease.

The second part addressed was the involvement of lymphocytes in the pathogenesis of Akabane disease. Although the role of the lymphoid system is usually considered to be one of protection against infections (Berger et al, 1981), it has been suggested that some viruses may replicate in lymphoid cells and that lymphoid structures may form an important pathway for viral spread to target organs (Bodian, 1955; Fraser and Martin, 1978). In view of this suggested role of lymphoid cells in the spread of these other viruses, it seemed important to investigate the circulating lymphocyte as a mechanism for spread of Akabane virus.

In this study, lymphocytes from sheep foetuses directly infected with Akabane virus were collected daily and examined by electron microscopy for the presence and location of virions, in order to study whether an apparently non-neoplastic and non-persistent virus could replicate and/or survive in foetal lymphocytes.

7.2 EXPERIMENTAL DESIGN

This chapter describes a third variant of exposure of foetal lambs to Akabane virus. In this instance older foetuses were infected subcutaneously with the virus, and measurements of the foetal response were undertaken at intervals thereafter. Prescapular efferent lymphatic cannulations were performed on all of these foetuses, while jugular venous cannulations were also undertaken on two of them. As in the two previous

studies, the distribution of the virus, the effect of the infection upon the host, and the host immune response were investigated.

The prescapular efferent lymph vessels of eight foetuses between 104 and 126 days gestation were cannulated as described in Materials and Methods. Two of these foetuses were used as controls - one, (at day 125) being left unchallenged, and the other (at day 131) being challenged with 0.2 ml of medium containing Vero cells, administered S/C in the region anterior to and drained by the prescapular lymph node. A further foetus (at day 125) was challenged similarly with 0.2 ml of medium containing 2.0×10^6 PFU of Akabane virus which had been inactivated as a control to separate the influence of viral replication from that of the antigens present on the immunogenicity and pathogenicity of the inoculum. The remaining five were challenged similarly between day 104 and day 126 with 0.2 ml of medium containing 2.0×10^6 PFU of viable Akabane virus and recovered for examination 14 - 21 days later. Efferent lymph was collected continuously, and measurements of volume, rate of flow and cell content made each 12 hours, giving a series of observations that could be viewed against time. The jugular vein of two of these foetuses (day 108 and day 126) was also cannulated and blood samples were taken at 48-hour intervals for up to 17 days. Lymphatic cannulation was attempted on a further 12 foetuses, but these were not included here because lymph flowed for less than three days. Reasons for these failures included death of the ewe or foetus due to disease, anaesthetic loss, and an apparent increase in foetal susceptibility to surgical handling after 125 days

gestation. Problems associated with the indwelling cannula included its rupture, displacement and kinking, and clotting of lymph within the cannulae. The immune response was directly studied, using the presence of specific antibody and alteration in the number of cells bearing surface immunoglobulin as indicators of humoral immune function, and, as with the adults in Chapter Three, lymphocyte numbers and their in vitro reactivity as indicators of cell-mediated immunological function. While full assessment of all parameters could not be achieved in all animals, the lymph was examined for -

(a) Flow rate.

(b) Specific neutralizing antibody level (estimated by the plaque-inhibition test).

(c) The percentage of efferent lymphocytes bearing surface immunoglobulin (detected by immunofluorescent staining).

(d) Efferent lymph cell output and type.

(e) In vitro reactivity of efferent lymphocytes to Con A and to Akabane virus (assessed by lymphocyte transformation assay).

Lymphocyte transformation assays (described in Materials and Methods) were performed in vitro at two to five day intervals, in order to follow the kinetics of the local cellular immune response. Efferent lymphocytes from one unchallenged, one Vero-challenged control and four viable virus-challenged foetuses were also examined by electron microscopy for the presence of Akabane virus, and by plaque assay for Akabane virus titre, in order to determine whether they played any role in the spread of the virus.

The serial blood samples collected from the two cannulated foetuses were used for assay of specific neutralizing antibody concentration, and for white blood cell and differential leukocyte counts.

The seven foetuses which survived until retrieval were examined for gross abnormalities, and the following tissues were fixed for histological examination: brain, spinal cord, skeletal muscle, liver, spleen, thymus, kidney, adrenal, thyroid, lung, foetal membranes, placentome and bone. Efferent lymphocytes from two control and four infected foetuses were also examined by electron microscopy.

Selected tissues (brain, spinal cord skeletal muscle, allantoic and amniotic fluids and membranes, placentome, liver, spleen, thymus and blood) from these seven foetuses were also examined by plaque assay titration for the presence and titre of viable Akabane virus, and by immunofluorescent microscopy for localization of Akabane antigen.

The ewes were bled at various times after foetal cannulation, and the sera assayed for specific neutralizing antibody concentration.

7.3 RESULTS

No lymph was obtained from #75 due to blockage of the cannula.

Virus distribution

Table 7.3 incorporates the results of viral titrations and viral antigen immunofluorescent studies on tissues of cannulated foetuses. Virus was not recovered, nor was viral

TABLE 7.1 - Akabane virus titre (PFU/ml) and presence of virions in efferent lymphocytes and blood of cannulated foetuses.

		Presence of virions[1]				Titre of viable virus[2]						
		Time (d) post-inoculation				Time (d) post-inoculation						
Foetus	Tissue	1	3	5	8	1	2	3	4	5	11	13
61[a]	EL	-	-	-	-		-			-		
20[b]	EL	-	-	-	-		-			-		
16[c]	EL						-			-		
69[e]	EL	+	+	+	+	5.0			-	-		
75[e]	EL											
95[e]	EL	-	-	-								
64[e]	EL	-	-	-		$5.0x10^2$	$5.0x10^1$			$5.0x10^1$		
63[e]	EL			-	-	$1.0x10^1$	$3.0x10^1$	$2.0x10^1$				
	BLOOD					-		5.0		$1.5x10^1$	-	-

1 = cells were examined for the presence of virions by electron microscopy
2 = viable virus was titrated in separated and washed efferent lymphocytes and in whole blood by the plaque assay.

EL = efferent lymphocytes; a = foetus not challenged; b = foetus challenged with Vero antigen;

c = foetus challenged with inactivated Akabane virus; e = foetus challenged with viable Akabane virus;

- = virions/viable virus not detected; + = virions present.

TABLE 7.2 - Akabane virus titres (PFU/ml) in cultured normal foetal efferent lymphocytes at intervals after addition of viable virus

Duration of culture (d)	Foetus 61 (aged 133d)		Foetus 20 (aged 136d)	
	-Akabane	+Akabane	-Akabane	+Akabane
0	-	2.0×10^{5}*	-	2.5×10^{5}*
2	-	1.0×10^{5}	-	1.0×10^{5}
4	-	1.0×10^{4}	-	2.5×10^{4}
6		1.0×10^{3}	-	5.0×10^{3}

* = titre of virus added to the cultures.

+ Akabane = viable virus was added to the cultures at Day 0.

- Akabane = viable virus was not added to the cultures.

TABLE 7.3 - Distribution of Akabane virus and viral antigen in cannulated foetuses

	Titre of virus								Viral antigen							
Sheep	61[c]	20[g]	16[e]	69[a,f]	75[f]	95[f]	64[f]	63[f]	61[c]	20[g]	16[e]	69[a,f]	75[f]	95[f]	64[b,f]	63[f]
Age (d) at inoculation	125	131	134	104	108	109	123	126	125	131	134	104	108	109	123	126
Age (d) at recovery	141	144	148	121	125	122	147	143	141	144	148	121	125	122	147	143
Cerebrum		-	-		-	-	-	-		-	-		+	+		+
Placentome		-				$5.0x10^2$		-		-				+		
Allantoic membrane		-	-			-		-		-	-		+	-		+
Amniotic membrane						-		-								
Skeletal muscle		-	-		-	-	-	-		-	-		-	-		-
Spleen					-	-		-					-			-
Thymus						-	-						-			-
Spinal cord	-				-		-	-	-				+			+
Blood	-				-	-		-								
Allantoic fluid						-										
Liver					-								-			-

a = foetus died *in utero*, no samples obtained; b = no samples taken for immunofluorescent studies;

c = foetus not challenged, nor autopsied because it was used in a further experiment (Chapter 4);

g = foetus challenged with Vero antigen; e = foetus challenged with inactivated Akabane virus;

f = foetus challenged with viable Akabane virus.

antigen detected, in any of the tissues from the control foetuses that were examined. Of the three foetuses, tissues from which were examined 13 to 17 days after inoculation with viable virus, all had viral antigen in the brain (cerebral cortex) and spinal cord. Foetal membrane and placentome were less consistently positive. The cell types in which antigen was detected were the same as those described in Chapter Five in younger foetuses, namely trophoblast, neurones, glial cells, and cerebral vascular endothelium (Plate 7.1).

The titre of viable virus was assayed in tissues from four of the five infected foetuses 13 to 17 days after inoculation. Virus was detected only in placentomes of one foetus. Blood from a second (#63), although negative at autopsy, had previously been positive for virus (Table 7.1). This failure to recover viable virus from foetal tissues other than placentome contrasts with the wide distribution of viral antigen detected by immunofluorescent microscopy.

Specific antibody

The specific neutralizing antibody titres of foetal sera and foetal fluids are shown in Table 7.4. Specific antibody could not be detected in serum or lymph of any of the foetuses left unchallenged or challenged with Vero control or inactivated Akabane virus. Of the foetuses challenged with viable virus, specific antibody appeared in sera and/or lymph of the four whose cannulae flowed for sufficiently long, that is between eight and eleven days post-inoculation. Flow ceased from the cannula of the fifth foetus (#64) after five days. This lamb had a serum antibody titre of 11 mm when tested

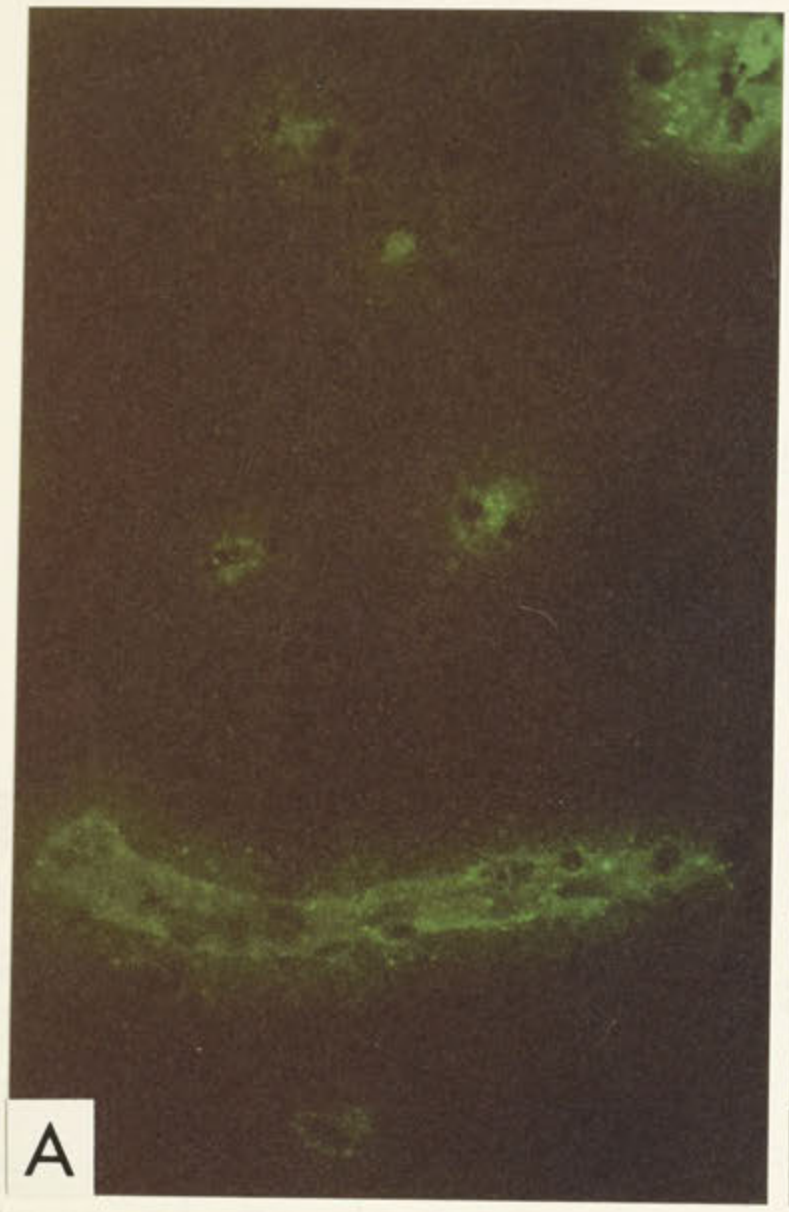
PLATE 7.1 Fluorescein-labelled Akabane antigen in tissues of subcutaneously infected foetuses.

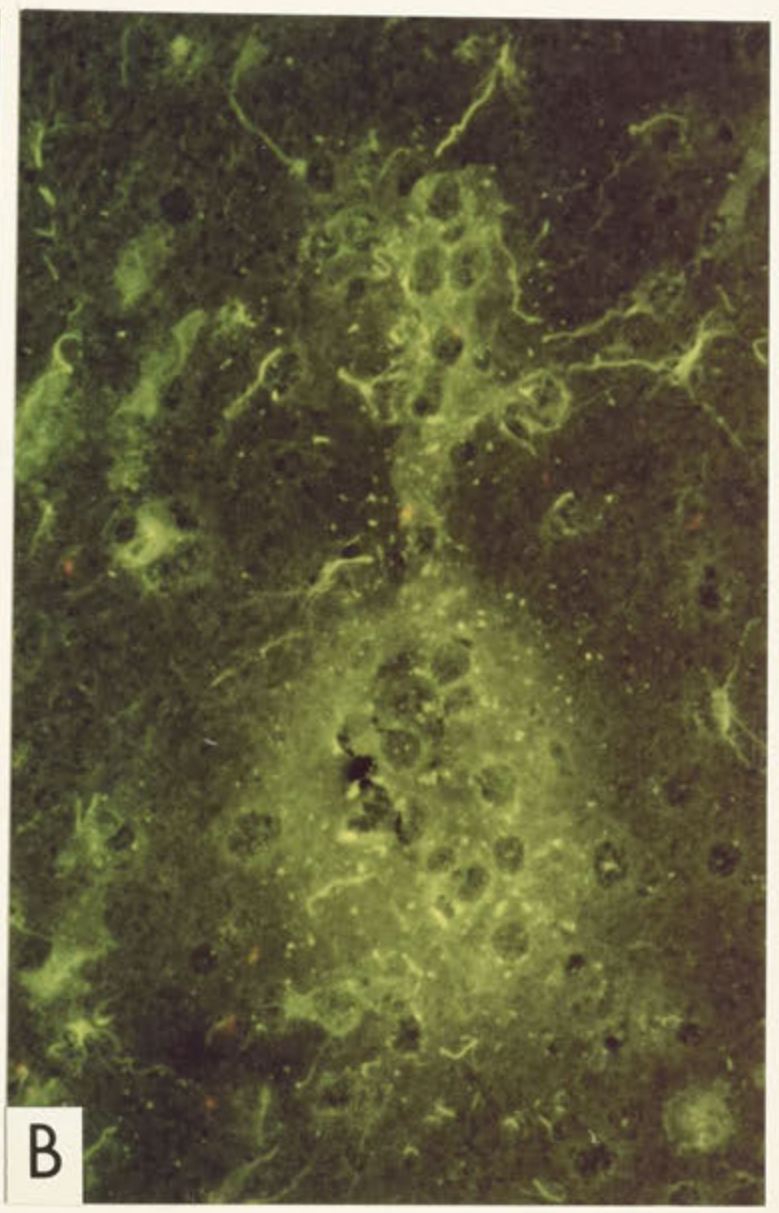
A: # 63, aged 143d; cerebrum. Fluorescein has specifically labelled cytoplasm of scattered and grouped parenchymal cells, and of vascular endothelial cells.
This section was counterstained with Evans Blue.
x 250.

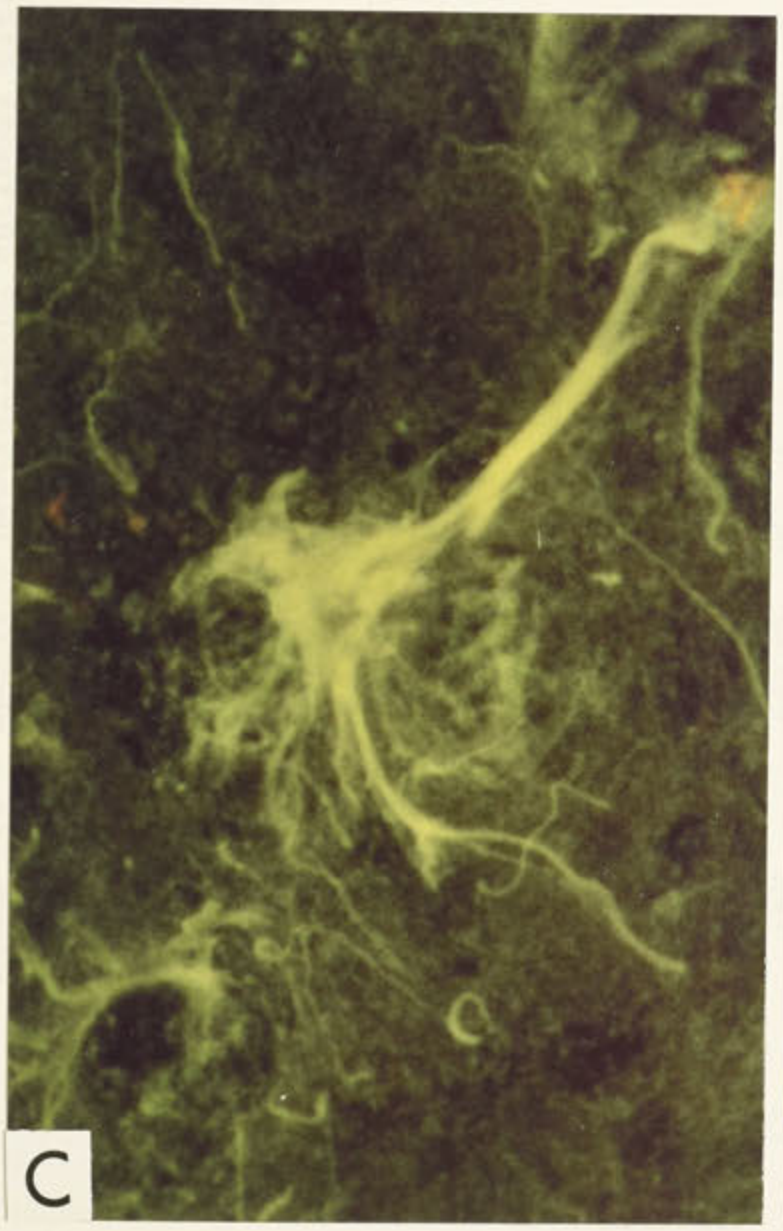
B: # 95, aged 122d; cerebrum. Fluorescein has specifically labelled cytoplasm of scattered and grouped parenchymal cells. Positively stained cell processes are visible.
x 250.

C: # 95, aged 122d; cerebrum. A high power view of a glial cell, showing labelling of the viral antigen in the cell processes.
x 1000.

D: # 95, aged 122d; placentome. An unusually large number of cells labelled specifically for viral antigen; this specimen also showed more severe necrosis than did placentomes of other sheep.
x 250.


A


B


C

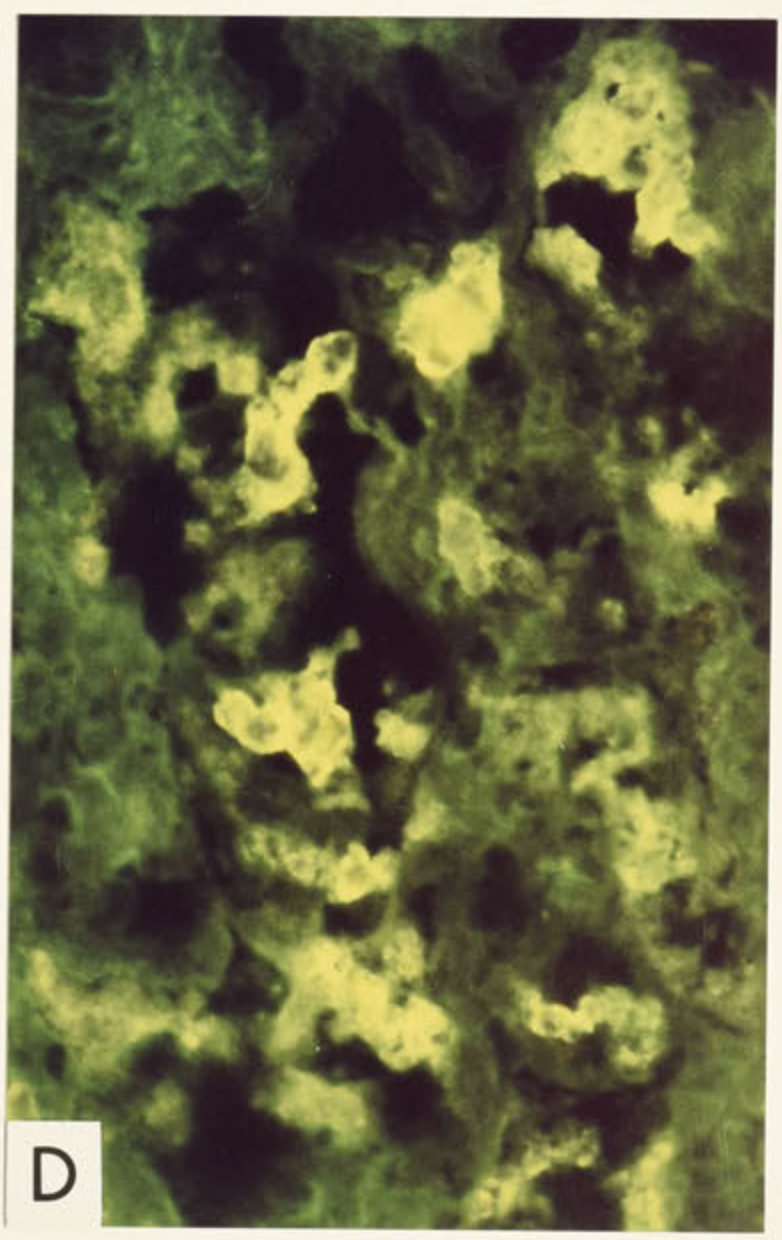

D

TABLE 7.4 - Specific neutralizing antibody titres(mm) in serum and lymph of cannulated foetuses.

Foetus	Foetal age (d) at inoculation	Fluid	Time (d) after inoculation																	
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
61[a]	125	lymph	0							0									0	
20[b]	131	lymph	0							0						0				
16c	134	lymph	0							0							0			
69[e]	104	lymph	0			0	0	0	0	0		0	0	6						
75[e]	108	serum	0	0		0		0		0		11		15				16		
95[e]	109	lymph	0		0			0		0	9									
		serum															7			
64[e]	123	lymph	0	0	0	0	0	0												
63[e]	126	lymph	0	0	0	0	0	0	0	0	4	10	14	12	5	7	9	5		
		serum	0	0	0	0	0	0	0	0	0	9		5		11		8		

Antibody titre is expressed as the diameter (mm) of the zone of inhibition.

a = foetus not challenged; b = foetus challenged with Vero antigen;

c = foetus challenged with inactivated Akabane virus; e = foetus challenged with viable Akabane virus.

64 - this lamb had seroconverted by D 3 after birth, i.e. 25 d after inoculation.

three days after birth.

The specific neutralizing antibody concentrations of maternal sera at intervals after foetal inoculation with the virus are shown in Table 7.5. All five adults seroconverted, at least one of them doing so by five days after foetal infection. Ewe #69 seroconverted six days before specific antibody was detected in the lymph of her foetus.

Pathology

Table 7.6 summarized the pathological findings in these cannulated foetuses. No. 69 died and autolysed *in utero*, and #61 was used in a later experiment and so was not autopsied. No samples were subsequently obtained from these foetuses. None of the remaining foetuses had developed macroscopically visible lesions. The only foetuses in which histological lesions were detected were those infected with viable Akabane virus. Pathological changes were apparent on microscopic examination in the brain and skeletal muscle of two of the four surviving S/C infected foetuses. In the case of the less severely affected lamb, the cerebrum and midbrain contained areas of focal necrosis, malacia and cavitation, together with mild perivascular cuffing. In the cerebellum of this lamb, the remaining Purkinje cells were generally of necrotic appearance (Plate 7.2). There was a mild mononuclear infiltration of the meninges whilst focal necrosis, especially of sarcoplasm, was evident in skeletal muscle. Apart from an increase in the extent of the splenic white pulp, other tissues appeared to be normal.

The more severely affected lamb (#95) had severe peri-

TABLE 7.5 - Specific neutralizing antibody titres(mm) in serum of pregnant ewes after injection of Akabane virus into the foetus.

Ewe	Foetal age (d) at inoculation	Time (d) after inoculation 0	5	7	10	15	17
61[a]	125	0				0	
20[b]	131	0			0		
16[c]	134	0				0	
69[e]	104	0	3		5		4
75[e]	108	0					6
95[e]	109	0	0	9			
64[e]	123	0	0		14	20	21
63[e]	126	0					18

Antibody titre is expressed as the diameter (mm) of the zone of inhibition.

a = foetus not challenged.

b = foetus challenged with Vero antigen.

c = foetus challenged with inactivated Akabane virus.

e = foetus challenged with viable Akabane virus.

TABLE 7.6 - Gross and microscopic abnormalities of foetuses challenged subcutaneously.

Sheep	20[a]	16[b]	69[c,e]	75[c]	95[c]	64[c]	63[c]
Age (d) at inoculation	131	134	104	108	109	123	126
Age (d) at recovery	144	148	121	125	122	147	143
Gross Pathology	-	-		-	-	-	-
Histopathology:							
Cerebrum	-	-		++	+++		-
Cerebellum	-	-		+	-	-	-
Spinal cord	-	-		-	-	-	-
Meninges	-	-		+	++	-	-
CNS infiltration	-	-		+	++	-	-
Membrane infiltration	-	-		+	+	-	-
Muscle infiltration	-	-		-	+	-	-
Placentome	-	-			+++	-	
Skeletal muscle	-	-		+	+	-	-
Foetal membrane	-	-			+	-	-

Abnormalities were scored (-) - (++++), where (++++) = total loss of tissue.

a = foetus challenged with Vero antigen.

b = foetus challenged with inactivated Akabane virus.

c = foetus challenged with viable Akabane virus.

e = foetus died in utero, no samples were obtained.

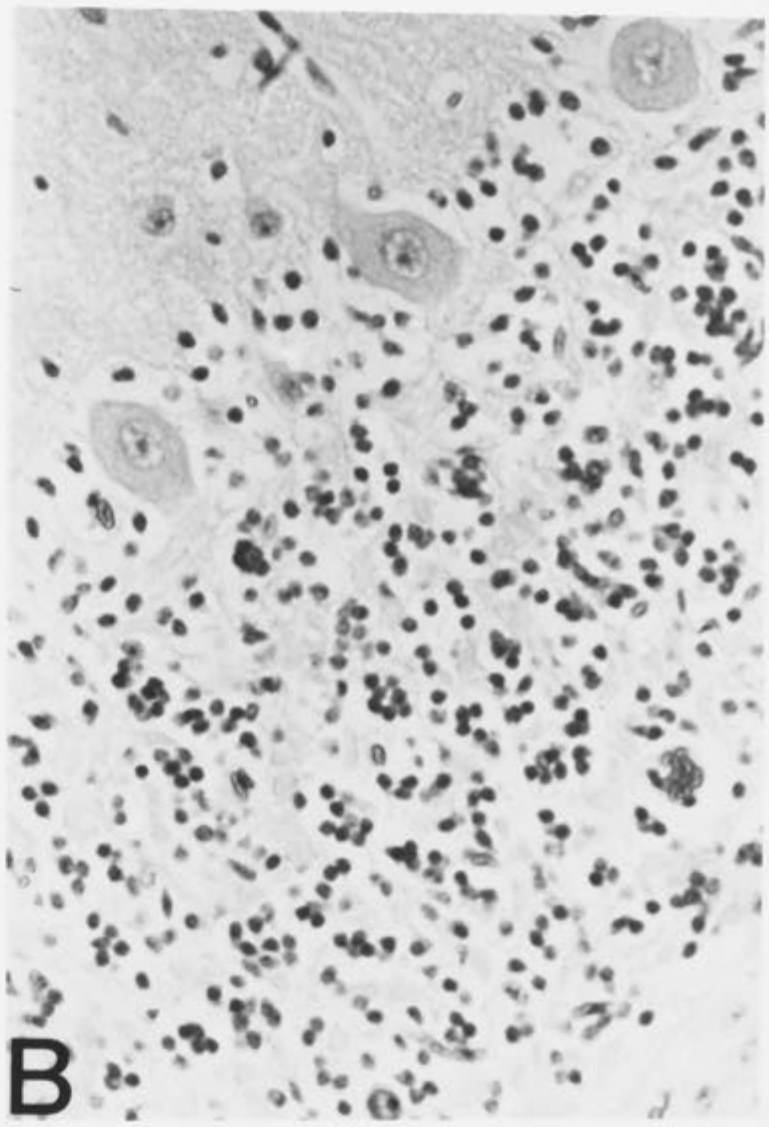
PLATE 7.2 Microscopical lesions of foetal cerebellum after foetal infection with Akabane virus.

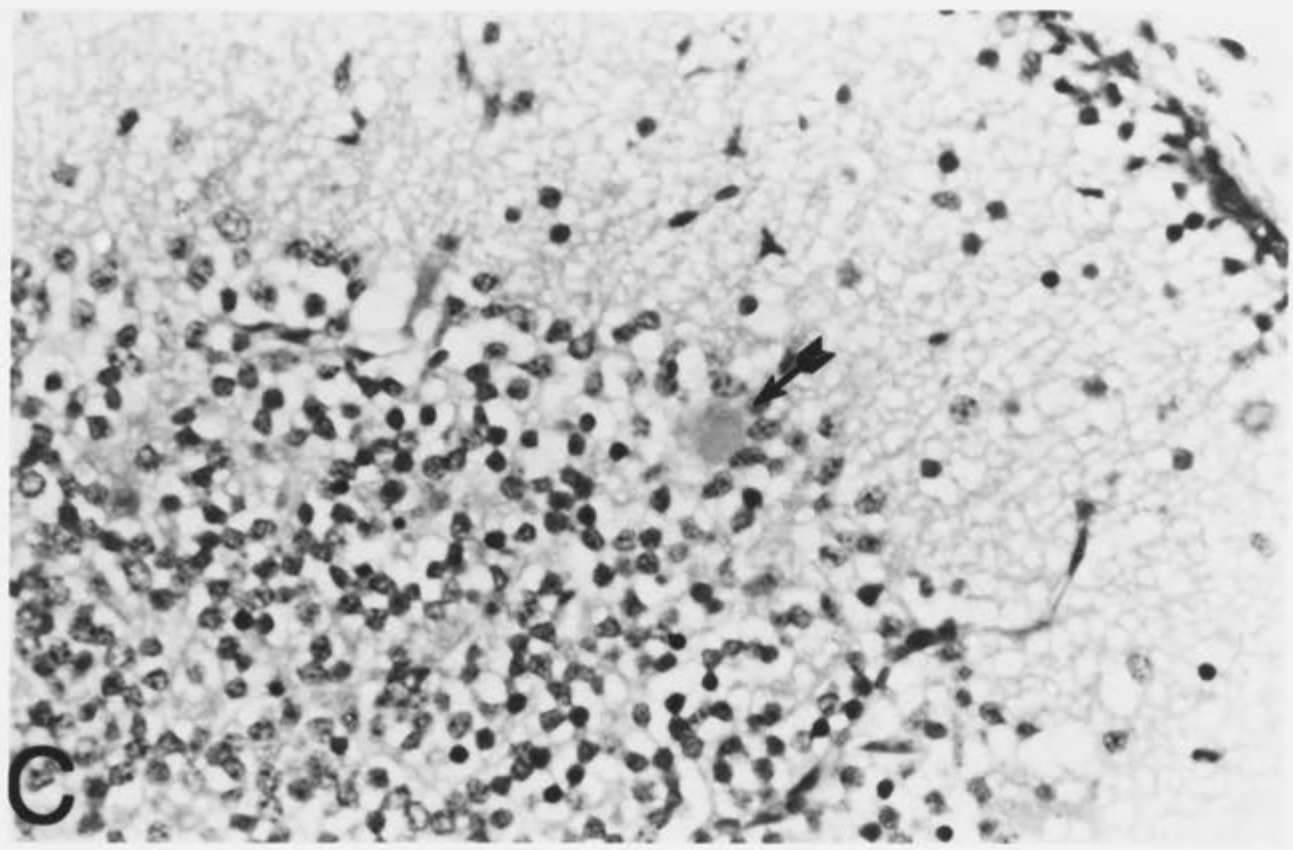
A: Section of a lobe of the cerebellum of a control foetus aged 144d, 14 d after subcutaneous inoculation with Vero antigen, showing all cell layers.
Haematoxylin and eosin, x 100.

B: A higher-power view of the same section, showing the normal Purkinje cell layer.
Haematoxylin and eosin, x 250.

C: Section of a lobe of the cerebellum of a foetus aged 125d, 14d after subcutaneous inoculation with viable Akabane virus, showing the area normally occupied by the Purkinje cell layer. Purkinje cells are absent, with the exception of one necrotic cell (arrowed).
Haematoxylin and eosin, x 250.


A


B


C

vascular cuffing and infiltration by mononuclear cells in the cerebral hemispheres, midbrain and meninges, with local lymphocyte infiltration in skeletal muscle, lung and foetal membrane. The meninges were grossly thickened and hyperaemic, and cerebrum and midbrain contained areas of severe focal necrosis and swollen endothelial cells. There were also foci of mild necrosis in skeletal muscle. The prescapular lymph node had well-developed germinal centres. Foetal membranes were oedematous, and the placentomes were extremely necrotic (Plate 6.5). Both foetal and maternal components of the placentome were involved, but maternal cotyledons appeared to be more severely damaged, with widespread liquefactive necrosis and mononucleocyte infiltration. Other tissues appeared to be normal.

Efferent lymph data

The efferent lymph flow rates and cell outputs from cannulated lymph nodes are shown in Tables 7.7 to 7.13 and illustrated in Figures 7.1 to 7.7. Efferent lymph flow rate remained constant for all control and challenged foetuses except for #95, where there was a slight rise in flow rate accompanying the rise in cell output (Fig. 7.7).

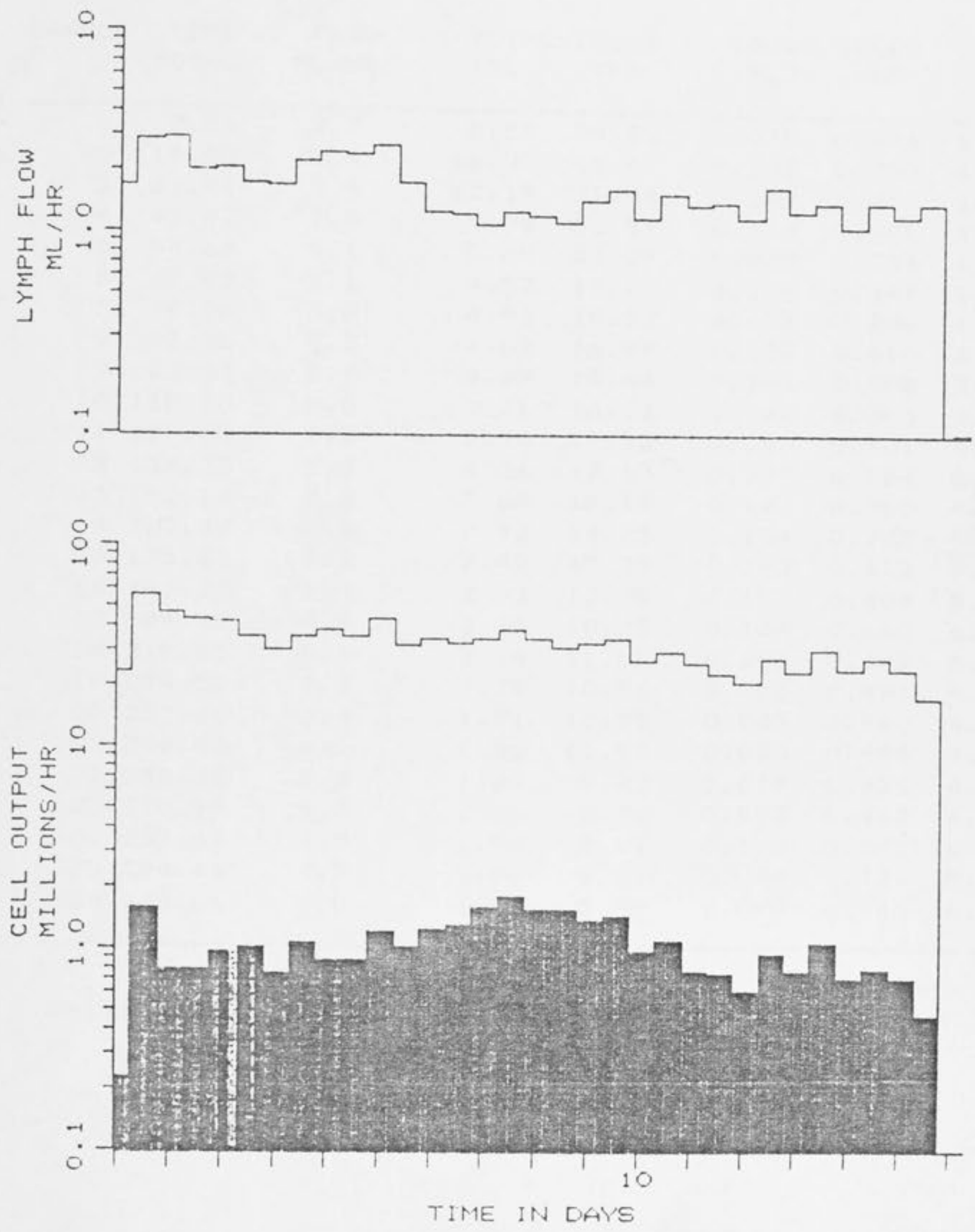
Total cell output from control lymph nodes (Figs. 7.1 to 7.3) fell gradually and consistently as collection continued, probably due to foetal failure to generate lymphocytes sufficiently rapidly to replace all that were removed in the draining lymph. This assumption is supported by the fact that inversion of the lymphocyte:neutrophil ratio (Fig. 7.11) was observed in the peripheral blood of those infected foetuses which were

TABLE 7.7 - Efferent lymph data from foetus # 61, unchallenged.

SAMPLE	TIME TOTAL	FLOW ML/HR	TOTAL CELLS /ML	TOTAL CELLS /HR	LARGE CELLS /ML	LARGE CELLS /HR	%
1	7.25	1.7	13.77	24.05	0.134	0.234	0.97
2	20.00	2.9	19.89	59.03	0.550	1.634	2.76
3	31.25	3.0	15.58	47.74	0.261	0.801	1.67
4	44.00	2.0	21.19	44.27	0.391	0.817	1.84
5	55.50	2.1	20.40	43.78	0.461	0.990	2.26
6	68.25	1.7	20.27	36.15	0.581	1.036	2.86
7	80.00	1.7	17.52	30.99	0.433	0.767	2.47
8	92.33	2.3	15.42	35.95	0.466	1.087	3.02
9	104.33	2.5	15.10	38.65	0.346	0.886	2.29
10	116.49	2.4	14.54	36.30	0.360	0.900	2.48
11	128.41	2.7	16.02	44.46	0.442	1.228	2.76
12	139.99	1.8	18.25	32.95	0.578	1.044	3.17
13	151.99	1.3	27.35	35.56	0.991	1.289	3.62
14	163.99	1.2	26.54	33.83	1.037	1.323	3.90
15	175.83	1.1	31.52	34.96	1.484	1.646	4.71
16	187.99	1.2	29.82	38.66	1.420	1.841	4.76
17	199.99	1.2	28.65	35.81	1.291	1.614	4.50
18	211.99	1.1	28.17	32.39	1.400	1.610	4.97
19	223.99	1.4	23.10	33.88	0.949	1.392	4.10
20	236.33	1.6	20.77	35.12	0.884	1.495	4.25
21	248.24	1.2	22.68	27.34	0.831	1.001	3.66
22	260.66	1.5	19.10	30.28	0.711	1.128	3.72
23	272.24	1.3	19.31	26.86	0.574	0.799	2.97
24	284.24	1.4	16.45	23.86	0.527	0.765	3.20
25	296.74	1.2	17.53	21.22	0.515	0.624	2.94
26	308.41	1.7	16.50	28.36	0.562	0.966	3.40
27	320.33	1.3	18.79	24.54	0.601	0.786	3.20
28	331.99	1.4	22.06	31.49	0.771	1.101	3.49
29	343.99	1.0	23.04	24.97	0.684	0.741	2.97
30	355.99	1.4	19.75	28.31	0.560	0.802	2.83
31	368.16	1.2	19.88	24.64	0.594	0.736	2.99
32	379.99	1.4	12.63	18.07	0.331	0.474	2.62

cells x 10^6

FIGURE 7.1 - Efferent lymph data from foetus # 61, unchallenged.



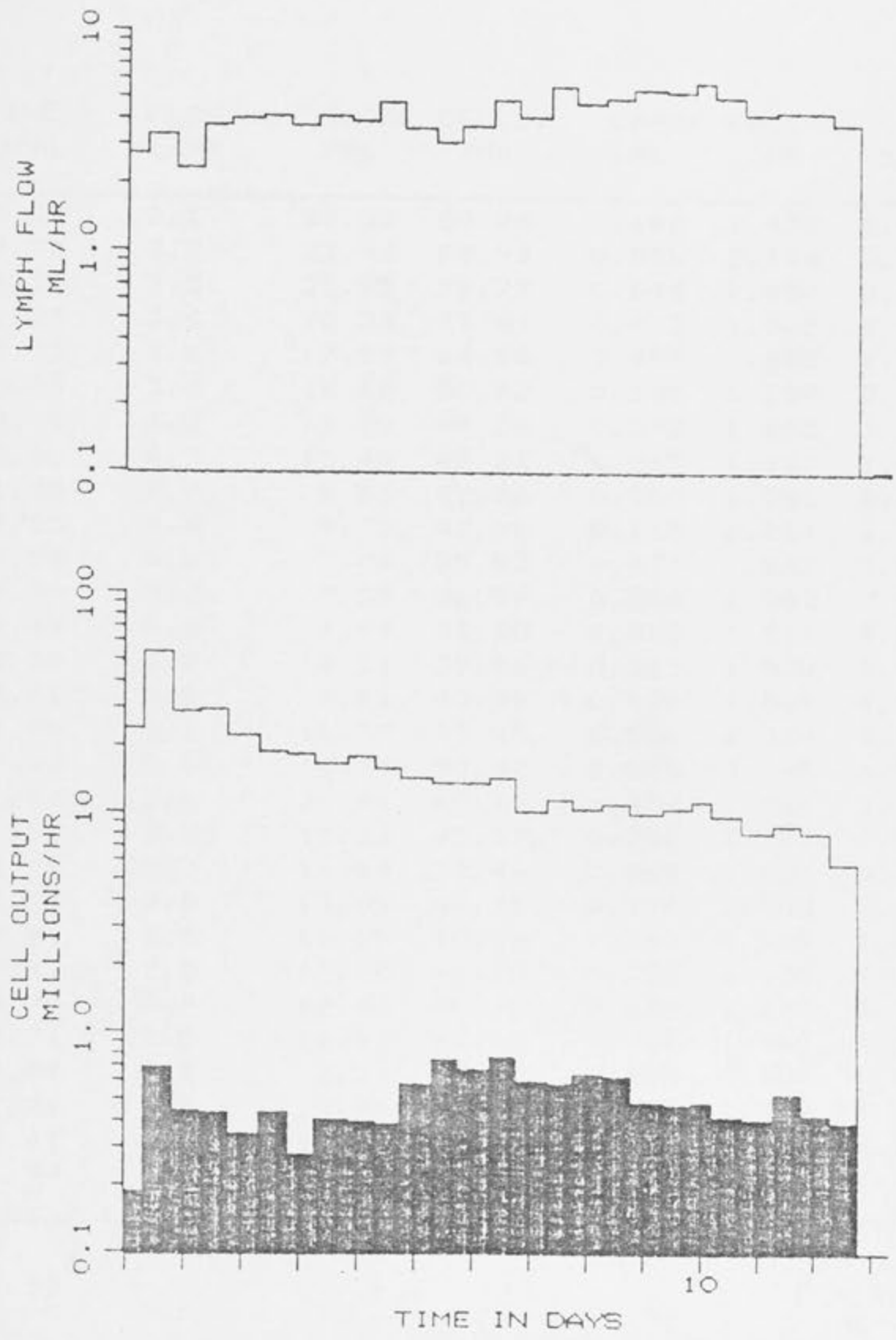
The hatched area represents the output of blast cells, while the open histogram represents total cell output.

TABLE 7.8 - Efferent lymphocyte data from foetus # 20, challenged at 131d with Vero antigen.

SAMPLE	TIME TOTAL	FLOW ML/HR	TOTAL CELLS /ML	TOTAL CELLS /HR	LARGE CELLS /ML	LARGE CELLS /HR	%
1	7.66	2.7	8.88	24.83	0.070	0.196	0.79
2	19.83	3.4	16.03	55.81	0.203	0.708	1.27
3	31.41	2.4	12.19	29.38	0.189	0.457	1.55
4	42.83	3.8	7.98	30.59	0.114	0.440	1.43
5	55.66	4.1	5.69	23.39	0.086	0.354	1.51
6	67.83	4.1	4.57	19.17	0.105	0.441	2.30
7	79.08	3.8	4.91	18.92	0.072	0.280	1.47
8	92.58	4.2	4.02	16.99	0.097	0.410	2.41
9	103.83	3.9	4.69	18.64	0.102	0.405	2.17
10	115.33	4.8	3.31	16.11	0.080	0.393	2.44
11	127.83	3.6	3.94	14.58	0.162	0.601	4.12
12	139.33	3.2	4.36	14.04	0.237	0.764	5.44
13	152.16	3.8	3.69	14.18	0.182	0.700	4.94
14	163.33	5.0	2.92	14.85	0.154	0.785	5.28
15	175.83	4.2	2.40	10.25	0.143	0.612	5.97
16	187.33	5.8	2.03	11.85	0.103	0.604	5.10
17	199.33	4.9	2.18	10.71	0.134	0.660	6.16
18	210.83	5.1	2.14	11.07	0.125	0.647	5.84
19	224.58	5.7	1.75	10.06	0.085	0.491	4.88
20	237.08	5.4	1.91	10.52	0.087	0.480	4.56
21	244.83	6.1	1.86	11.43	0.080	0.494	4.32
22	258.08	5.3	1.84	9.82	0.079	0.423	4.31
23	270.99	4.4	1.86	8.31	0.093	0.414	4.98
24	282.66	4.5	1.95	8.92	0.118	0.542	6.07
25	294.66	4.5	1.80	8.21	0.094	0.431	5.25
26	306.66	4.0	1.48	5.98	0.099	0.403	6.73

cells x 10^6

FIGURE 7.2 - Efferent lymph data from foetus # 20, challenged at 131d with Vero antigen.



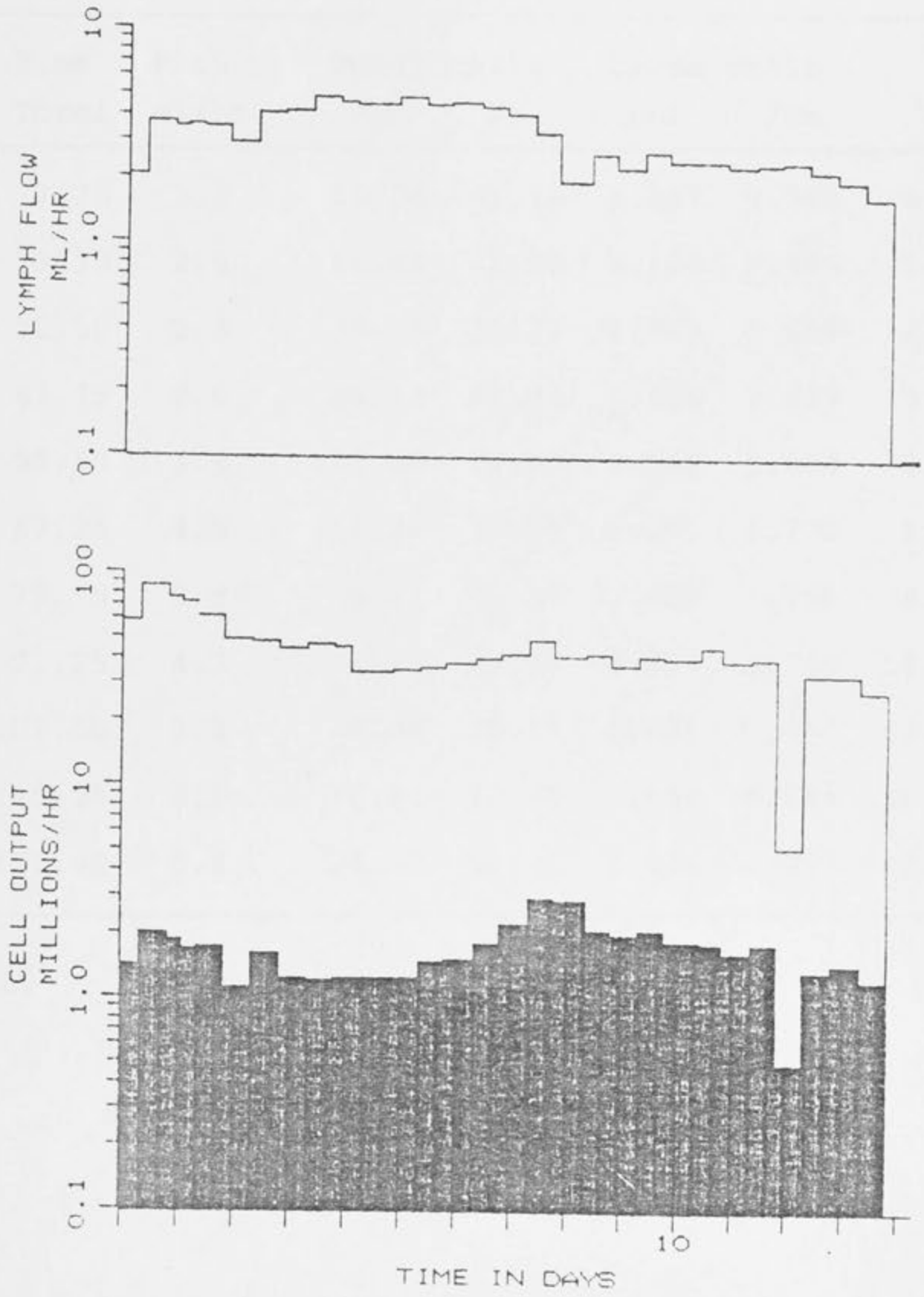
The foetus was challenged locally at day 0.
The hatched area represents the output of blast cells, while the open histogram represents total cell output.

TABLE 7.9 - Efferent lymph data from foetus # 16, challenged at 134d with inactivated Akabane virus.

SAMPLE	TIME TOTAL	FLOW ML/HR	TOTAL CELLS /ML	TOTAL CELLS /HR	LARGE CELLS /ML	LARGE CELLS /HR	%
1	8.25	2.1	28.38	60.94	0.688	1.478	2.42
2	19.75	3.7	23.46	88.92	0.558	2.118	2.38
3	26.25	3.5	21.95	78.77	0.544	1.954	2.48
4	32.25	3.6	20.58	75.81	0.473	1.745	2.30
5	43.75	3.6	17.99	64.90	0.499	1.802	2.77
6	56.00	3.0	16.68	50.82	0.380	1.159	2.28
7	68.00	4.2	11.70	49.25	0.392	1.653	3.35
8	80.00	4.3	10.48	45.24	0.293	1.267	2.80
9	91.75	4.9	9.53	47.56	0.250	1.251	2.63
10	99.25	4.8	9.73	47.08	0.265	1.284	2.72
11	117.08	4.6	7.74	35.83	0.273	1.267	3.53
12	129.08	5.0	7.29	36.59	0.255	1.282	3.50
13	139.99	4.6	7.94	37.20	0.322	1.511	4.06
14	152.58	4.7	8.21	38.81	0.325	1.538	3.96
15	163.91	4.5	8.91	40.80	0.408	1.869	4.58
16	175.99	4.1	10.37	43.45	0.560	2.348	5.40
17	187.41	3.4	14.59	50.42	0.896	3.098	6.14
18	200.74	2.0	20.91	42.18	1.470	2.966	7.03
19	211.58	2.7	15.62	43.37	0.780	2.165	4.99
20	223.99	2.3	16.29	38.44	0.869	2.051	5.33
21	235.33	2.8	14.88	41.75	0.764	2.146	5.13
22	250.91	2.5	16.19	40.96	0.747	1.889	4.61
23	259.91	2.5	17.94	46.15	0.722	1.858	4.02
24	272.74	2.4	16.67	40.10	0.694	1.671	4.16
25	283.74	2.5	16.60	41.60	0.745	1.867	4.48
26	295.99	2.5	2.04	5.21	0.196	0.502	9.62
27	307.74	2.3	14.96	34.49	0.587	1.354	3.92
28	320.41	2.0	16.53	34.33	0.711	1.476	4.29
29	331.99	1.7	16.66	29.08	0.709	1.238	4.25

cells x 10^6

FIGURE 7.3 - Efferent lymph data from foetus # 16, challenged at 134d with inactivated Akabane virus.



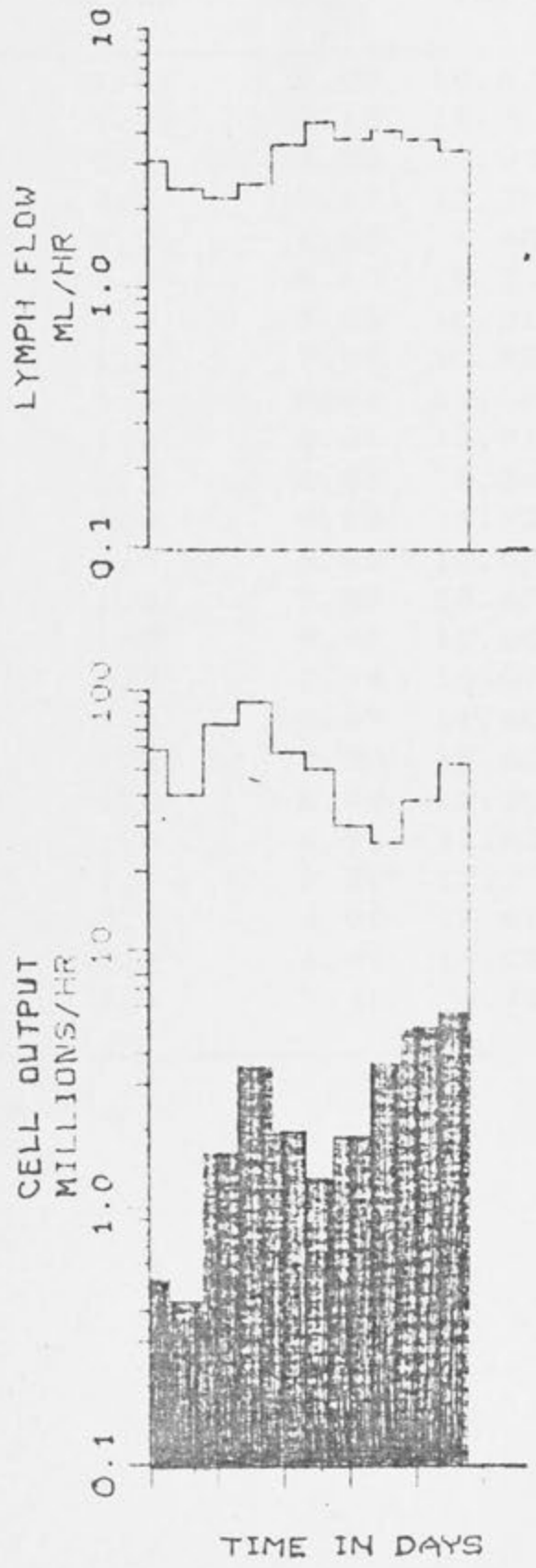
The foetus was challenged locally at day 0.
The hatched area represents the output of blast cells, while the open histogram represents total cell output.

TABLE 7.10 - Efferent lymph data from foetus # 64, challenged at 123d with viable Akabane virus.

Sample	Time Total	Flow ml/hr	Total cells /ml	Total cells /hr	Large cells /ml	Large cells /hr	Large cells %
1	7.25	3.2	19.08	61.16	0.167	0.538	0.88
2	19.50	2.4	16.84	41.52	0.180	0.445	1.07
3	31.50	2.3	33.31	77.77	0.710	1.659	2.13
4	43.75	2.6	36.14	94.21	1.396	3.639	3.86
5	55.50	3.6	16.20	59.62	0.560	2.064	3.46
6	67.25	4.5	11.38	51.76	0.291	1.322	2.55
7	79.75	3.8	8.11	31.36	0.505	1.956	6.23
8	91.25	4.2	6.23	26.61	0.874	3.730	14.02
9	103.50	3.8	9.98	38.77	1.352	5.250	13.54
10	115.25	3.5	15.21	53.90	1.651	5.848	10.85
11	128.00	0.4	24.72	11.41	2.614	1.207	10.57

cells x 10^6

FIGURE 7.4 - Efferent lymph data from foetus # 64, challenged at 123d with viable Akabane virus.



The foetus was challenged locally at day 0.
The hatched area represents the output of blast cells, while the open histogram represents total cell output.

TABLE 7.11 - Efferent lymph data from foetus # 69, challenged at 104d with viable Akabane virus.

SAMPLE	TIME TOTAL	FLOW ML/HR	TOTAL CELLS /ML	TOTAL CELLS /HR	LARGE CELLS /ML	LARGE CELLS /HR	%
1	6.50	1.8	5.83	10.63	0.127	0.232	2.18
2	18.50	1.9	6.10	11.70	0.269	0.516	4.41
3	30.50	0.9	8.38	7.83	0.278	0.259	3.31
4	42.50	2.2	5.87	13.39	0.427	0.974	7.28
5	57.75	2.3	4.05	9.48	0.339	0.793	8.36
6	66.75	2.0	4.60	9.20	0.176	0.353	3.83
7	78.50	1.7	5.96	10.21	0.527	0.902	8.83
8	90.50	1.5	7.05	10.92	0.550	0.851	7.79
9	102.50	1.3	8.81	12.06	1.005	1.376	11.40
10	114.50	1.6	8.61	13.91	0.790	1.275	9.16
11	119.50	1.2	6.56	8.36	0.998	1.271	15.20
12	126.50	1.3	8.82	11.73	0.820	1.089	9.28
13	138.50	1.5	6.66	10.07	0.683	1.033	10.25
14	150.25	1.3	7.89	10.67	0.885	1.196	11.20
15	162.25	1.2	9.41	12.00	1.279	1.632	13.59
16	174.91	1.3	7.94	10.97	0.327	0.452	4.12
17	187.24	1.3	8.69	11.60	0.323	0.431	3.71
18	198.91	1.6	7.50	12.63	0.193	0.325	2.57
19	210.74	1.8	6.48	12.30	0.168	0.320	2.60
20	223.41	1.6	6.91	11.63	0.188	0.317	2.72
21	235.41	1.7	7.29	12.79	0.198	0.347	2.71
22	249.16	2.1	6.00	12.66	0.119	0.252	1.99
23	259.66	2.2	6.41	14.25	0.169	0.376	2.64
24	270.16	1.1	5.68	6.78	0.163	0.194	2.87

cells x 10^6

FIGURE 7.5 - Efferent lymph data from foetus # 69, challenged at 104d with viable Akabane virus.

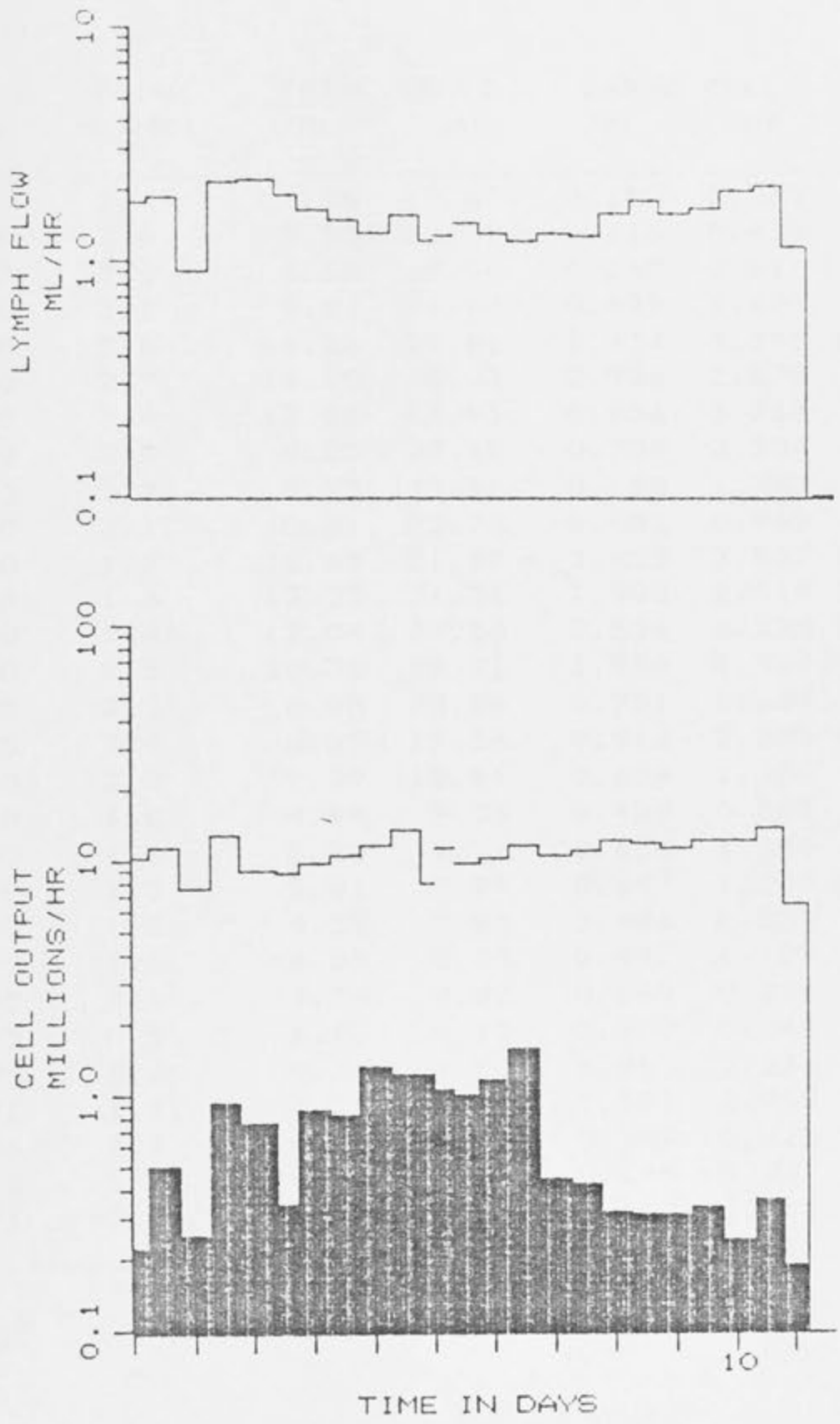


TABLE 7.12 - Efferent lymph data from foetus # 63, challenged at 126d with viable Akabane virus.

SAMPLE	TIME TOTAL	FLOW ML/HR	TOTAL CELLS /ML	TOTAL CELLS /HR	LARGE CELLS /ML	LARGE CELLS /HR	LARGE CELLS %
6	12.00	2.4	7.36	17.67	0.152	0.365	2.06
7	24.00	3.6	5.55	20.39	0.116	0.428	2.09
8	32.33	2.3	6.58	15.46	0.262	0.616	3.98
9	48.00	2.6	9.01	24.18	0.629	1.689	6.98
10	60.00	2.6	11.28	29.81	1.434	3.790	12.71
11	72.00	2.7	14.00	38.03	0.986	2.677	7.04
12	84.00	3.4	12.86	43.91	0.956	3.263	7.43
13	96.00	2.9	9.25	27.19	0.759	2.234	8.21
14	108.00	2.7	9.93	27.11	0.638	1.743	6.43
15	119.50	2.1	10.61	22.75	0.451	0.968	4.25
16	132.00	1.7	12.65	21.59	2.055	3.507	16.23
17	144.00	1.6	13.35	21.36	1.508	2.414	11.30
18	156.00	2.4	12.04	29.56	2.536	6.225	21.05
19	167.00	2.5	10.32	26.21	1.538	3.904	14.89
20	179.00	2.3	8.85	20.38	0.731	1.684	8.26
21	194.25	2.4	6.87	17.14	0.916	2.285	13.32
22	203.00	2.0	7.39	15.51	0.634	1.330	8.57
23	215.00	1.8	4.94	9.33	0.458	0.865	9.26
24	227.00	1.6	5.58	9.21	0.820	1.353	14.68
25	239.00	1.5	5.01	7.99	0.647	1.033	12.91
26	251.00	1.8	4.35	7.95	0.486	0.888	11.16
27	263.00	1.8	4.55	8.30	0.647	1.180	14.21
28	275.00	2.1	4.38	9.22	0.142	0.299	3.24
29	286.75	0.5	6.93	4.13	0.409	0.244	5.90
30	298.75	1.8	5.23	9.50	1.253	2.274	23.91
31	311.41	1.9	4.76	9.18	1.531	2.950	32.12
32	323.66	2.1	4.65	10.06	0.289	0.625	6.21
33	335.16	1.9	4.31	8.55	0.145	0.287	3.36
34	346.91	2.2	2.49	5.62	0.107	0.241	4.30

cells x 10^6

FIGURE 7.6 - Efferent lymph data from foetus # 63, challenged at 126d with viable Akabane virus.

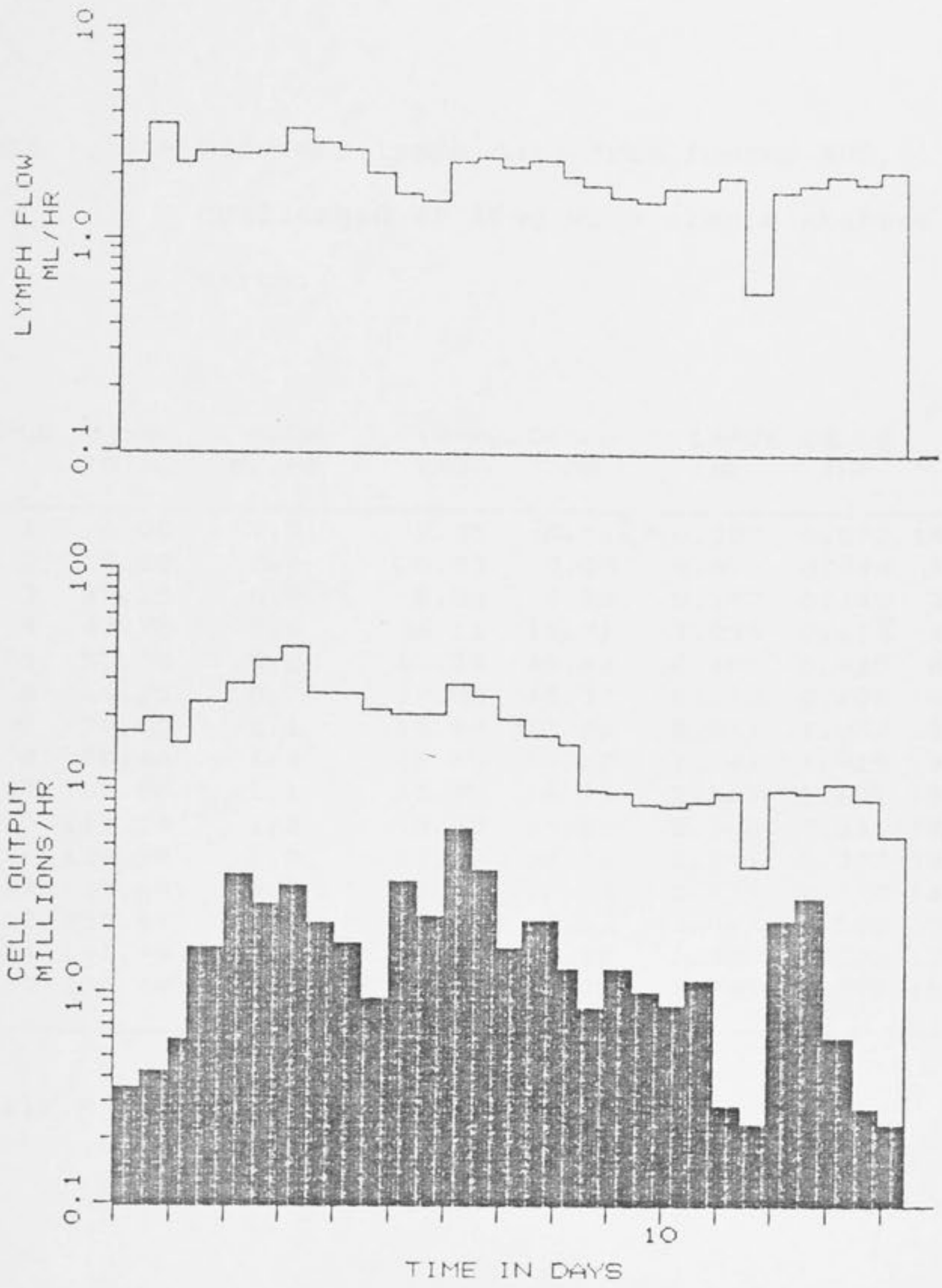
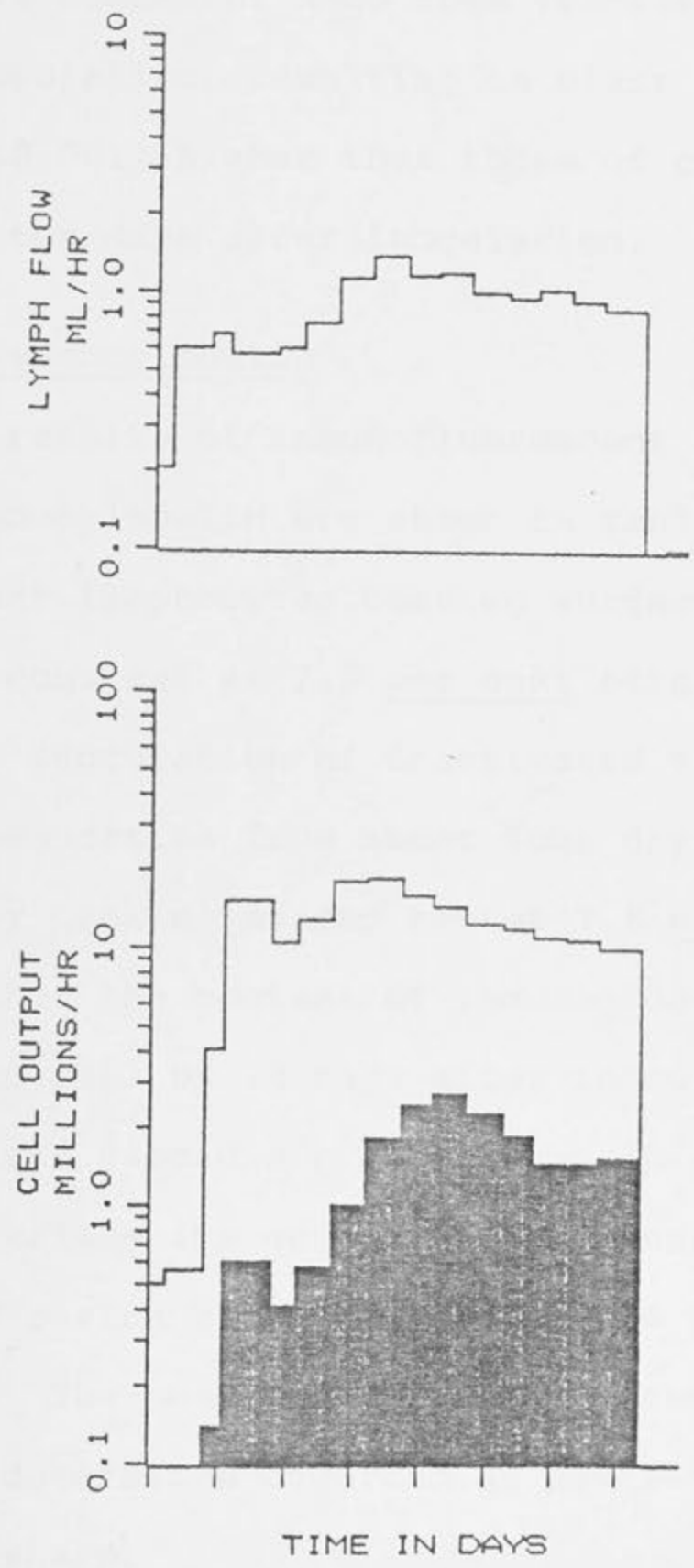


TABLE 7.13 - Efferent lymph data from foetus #95, challenged at 109d with viable Akabane virus.

SAMPLE	TIME TOTAL	FLOW ML/HR	TOTAL CELLS /ML	TOTAL CELLS /HR	LARGE CELLS /ML	LARGE CELLS /HR	%
1	6.00	0.2	2.35	0.51	0.333	0.072	14.15
2	19.50	0.6	0.93	0.58	0.071	0.044	7.67
3	27.25	0.7	5.86	4.22	0.197	0.142	3.36
4	43.75	0.6	26.11	15.71	1.055	0.634	4.04
5	53.00	0.6	17.38	10.82	0.685	0.427	3.94
6	66.25	0.7	17.05	13.33	0.773	0.605	4.53
7	78.00	1.1	15.84	18.86	0.871	1.037	5.49
8	90.66	1.4	13.80	19.61	1.347	1.915	9.76
9	102.00	1.1	13.95	16.74	2.167	2.601	15.53
10	114.16	1.2	12.35	15.20	2.300	2.831	18.62
11	126.99	1.0	12.29	12.91	2.264	2.377	18.41
12	138.49	0.9	12.32	12.30	1.977	1.973	16.04
13	150.49	1.0	10.90	11.53	1.419	1.502	13.02
14	161.99	0.9	11.75	11.32	1.580	1.522	13.44
15	177.49	0.8	11.51	10.30	1.785	1.598	15.50

cells x 10^6

FIGURE 7.7 - Efferent lymph data from foetus # 95, challenged at 109d with viable Akabane virus.



The foetus was challenged locally at day 0.
The hatched area represents the output of blast cells, while the open histogram represents total cell output.

subject to drainage of lymph, but not in those from which lymph was not collected. The total cell output from nodes challenged with live virus (Figs. 7.4 to 7.7) rose slightly from about two days after inoculation. This was accompanied by a rise in the blast cell proportion of the total cell output. This component also rose transiently from about two days after inoculation, resulting in blast cell counts significantly ($p<0.001$) higher than those of controls, and fell from seven to ten days after inoculation.

Surface immunoglobulin

The results of immunofluorescent labelling of surface-bound immunoglobulin are shown in Table 7.14. The proportion of efferent lymphocytes bearing surface-bound immunoglobulin remained constant at 2.5 per cent after challenge with Vero control. Inoculation of inactivated virus resulted in a rise in this proportion from about four days after inoculation, apparently peaking at day ten at 7.8 per cent. The results suggest that the content of immunoglobulin-bearing cells started to fall by 12 days after inoculation. Challenge with viable virus caused a greater rise in the proportion of cells bearing surface immunoglobulin, commencing at day three to four, and rising to an average of 8.6 per cent at approximately day five. The peak response of the two foetuses for which it could be determined occurred at day five, that is, the rise was very sharp.

In #69, the relative proportions of cells staining for immunoglobulin showed that on average, 6.1 per cent of the 8.0 per cent cells bearing immunoglobulin carried IgM, and that most of the increase in surface immunoglobulin following viral

TABLE 7.14 - Surface-bound immunoglobulin of foetal efferent lymphocytes

Foetus	20	16	63	64		69		95
Inoculum	Vero control	Inactive Akabane	Akabane	Akabane		Akabane		Akabane
Time post-inoculation(d)	% SIg	% SIg	% SIg	%SIg	%SIgM	%SIg	%SIgM	% SIg
1	1.2	2.4	2.6	4.1	3.1	6.2	4.0	
2			1.9	4.1				2.0
3			2.3	5.0		6.0	5.0	
4		5.1		3.7		8.0	6.2	
5			11.7	5.8		9.8	8.3	6.9
6	2.1	5.3	11.7			7.9	6.3	
7			5.5			8.9	6.9	
8	3.5	5.4	9.5					9.5
9			7.0					
10		7.8				7.7	5.1	
11			8.0			8.3	7.1	
12	3.5	6.5	9.9					
13			7.7					
14			6.1					
$\bar{x}$	2.5	5.4	7.0	4.5		8.0	6.1	6.1
n	4	6	12	5		8	8	3
SD	0.98	1.64	3.3	0.76		1.19	1.28	3.11

This table lists the percentage of efferent lymphocytes from locally challenged foetuses which stained positively for surface-bound immunoglobulins (SIg).

challenge consisted of IgM.

Lymphocyte transformation

The results of the in vitro lymphocyte transformation assays are shown in Tables 7.15 and 7.16 and Figures 7.9 and 7.10. Examination of efferent lymphocyte reactivity to Akabane virus revealed that cells (Table 7.16) draining from lymph nodes that had been challenged with live virus five days previously responded at significantly higher levels ($p<0.001$), than did cells from control foetuses (Table 7.15). Further, this response increased transiently with time after challenge, usually peaking about five days after inoculation (Fig. 7.10). One preparation continued to flow for 17 days, and this foetus (#63) had a second rise in response to Akabane virus at about 15 days after inoculation (Fig. 7.10).

Cells from all control and infected foetuses responded vigorously to the non-specific mitogens Con A and PHA (Tables 7.15 and 7.16). Except for a temporary depression in response of two of the four foetuses with Con A at three days after being injected with live virus, challenge, whether with live virus, inactivated virus or Vero antigen, resulted in a rise in this response to the mitogens with time after challenge, peaking at about day five, and in the case of #63, again at or after day 17 (Fig. 7.9).

Intra-lymphocytic Akabane virions

Efferent lymphocytes from two control and four infected foetuses were examined by electron microscopy for the presence of Akabane virions. Akabane virions are illustrated in Plates 7.3, 7.4 and 7.5. Table 7.1 summarizes the results of the

TABLE 7.15 - Response (^{3}H-thymidine incorporation) to Akabane virus and to Con A of efferent lymphocytes from control foetuses

Foetus	Age (d) inoculated	Time (d) post-inoculation	Controls		Non-specific stimulation		Specific stimulation	
			Medium Cpm	Vero Cpm	Con A Cpm	SI	Akabane Cpm	SI
61[a]	125		490	302	7680	15.7	384	1.3
20[b]	131	-1	125	142	66	0.5	78	0.5
		3.5	1102	3062	22286	20.2	1613	0.5
		6.5	251	587	78203	311.6	2227	0.4
		9.5	2910	1944	2147	0.7	2361	1.2
16[c]	134	-1	440	276	9592	21.8	314	1.1
		1	138	192	23389	169.5	253	1.3
		3	128	206	21501	168.0	123	0.6
		5	55	118	9556	173.7	92	0.8
		9	124	37	3237	26.1	103	2.8
		11	145	221	4084	28.2	163	0.7
		14	317	97	29044	91.6		

a = unchallenged; b = challenged locally with Vero control; c = challenged locally with inactivated Akabane virus.

Cpm = counts per minute of β emission from incorporated thymidine.

SI = stimulation index. SI for Con A = $\frac{\text{Cpm of cultures with Con A}}{\text{Cpm of cultures with medium only}}$

SI for virus = $\frac{\text{Cpm of cultures with virus}}{\text{Cpm of cultures with Vero}}$

In this and the two succeeding tables, the Cpm value is the mean of three replicates. Since standard errors did not exceed 12%, these are omitted for clarity. Indices greater than 2.0 were considered to indicate the presence of a response.

TABLE 7.16 - Response (^{3}H-thymidine incorporation) to Akabane virus and to mitogens of efferent lymphocytes from foetuses challenged locally with viable Akabane virus.

Foetus	Age (d) inoculated	Time(d) post-inoculation	Medium Cpm	Vero Cpm	Con A Cpm	SI	PHA Cpm	SI	Akabane Cpm	SI
64*	123	1	437	288	4957	11.3	13416	30.7	408	1.4
		3	232	491	109	0.5	32940	142.0	531	1.1
		5	231	514	22482	97.3	48147	208.4	1586	3.1
64+	123	1	410	452	5746	14.0	15225	37.1	476	1.1
		3	430	851	696	1.6	38386	89.3	976	1.2
		5	347	207	21595	62.2	49044	141.3	479	2.3
63*	126	1	212	219	4081	19.3	7373	34.8	305	1.4
		3	245	441	5976	24.4	25313	103.3	545	1.2
		5	200	209	23850	119.3	20301	101.5	817	3.9
		7	224	186	9738	43.4	11936	53.3	409	2.2
		9	466	196	3069	6.6	4719	10.1	190	1.0
		11	192	1479	5687	29.6	13916	72.5	703	0.5
		13	230	293	4541	19.7	11819	7.6	203	0.7
		15	130	191	3710	28.5	6053	46.6	631	3.3
		17	256	367	23248	90.8	22866	89.3	511	1.4
63+	126	1	270	216	2272	8.4	4375	16.2	130	0.6
		3	453	184	10141	22.4	31344	69.2	1127	6.1
		5	232	181	18928	81.6	21348	92.0	544	3.0
		7	294	310	15982	54.4	20146	68.5	268	0.9
		9	833	630	2371	2.8	2005	2.4	776	1.2
		11	748	1761	3313	4.4	3436	4.6	1571	0.9
		13	1558	592	4016	2.6	11819	7.6	305	0.5
69*	104	1	317	196	6746	21.3	5030	15.9	319	1.6
		3	234	1465	2043	8.7	7172	30.6	2201	1.5
		5	261	140	6291	24.1	11967	45.9	542	3.9
		7	132	1867	5472	41.5	8048	61.0	5239	2.8
		9	201	109	6638	33.0	10303	51.3	584	5.4
75*	108									
95*	109	1	196	97	2273	11.6			96	1.0
		5	709	2125	141624	199.8			10575	5.0
		7	500	1385	72603	145.2			5324	3.8

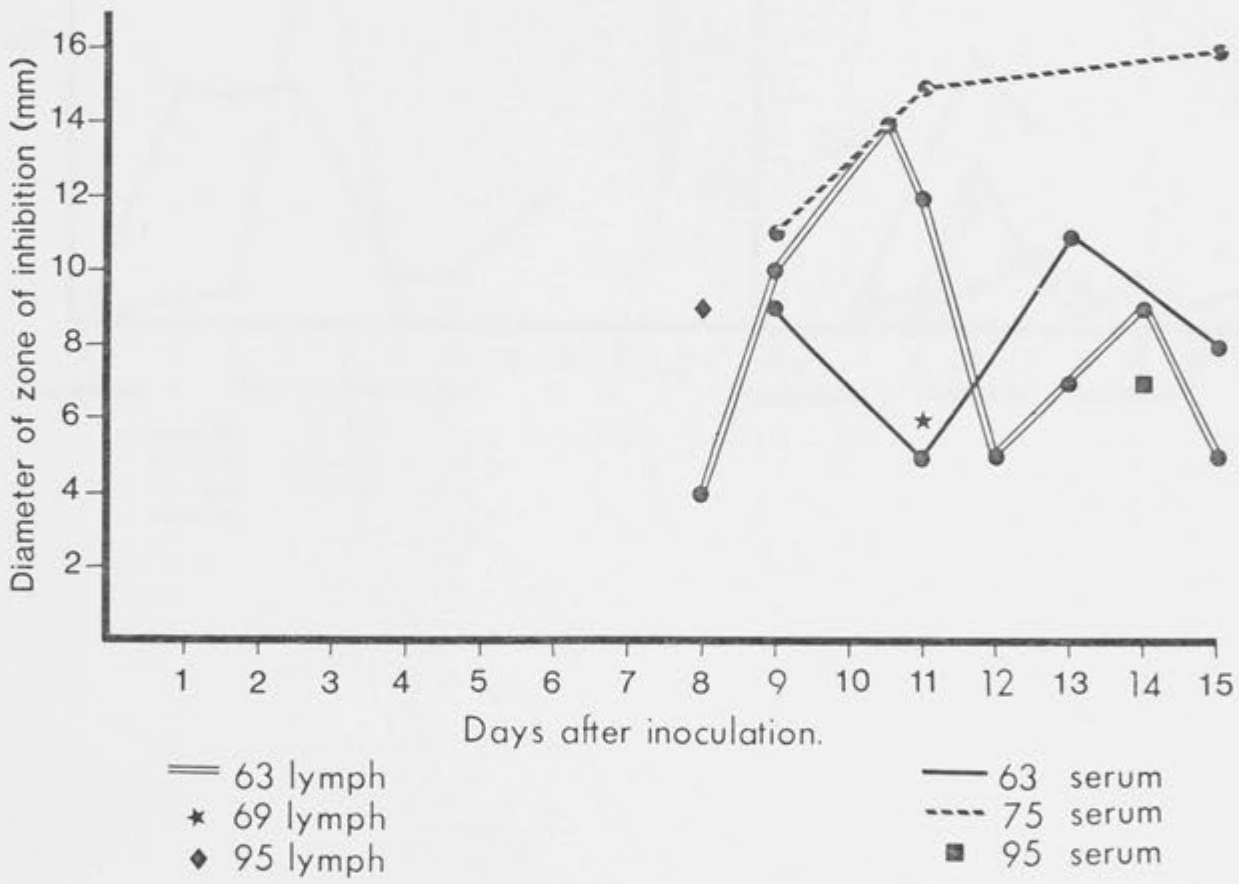
* = challenged side. + = unchallenged side. # 75 yielded no lymph.

Cpm = counts per minute of β emission from incorporated thymidine.

SI = stimulation index. SI for mitogens = $\frac{\text{Cpm of cultures with mitogen}}{\text{Cpm of cultures with medium only}}$

SI for virus = $\frac{\text{Cpm of cultures with virus}}{\text{Cpm of cultures with Vero}}$

Fig 7.8 Serum neutralizing antibody levels in lymph and serum of foetuses challenged locally with Akabane virus.



The numbers in the key refer to individual foetuses. Foetus #63 was inoculated at 126 days gestation, #69 at 104 days and #75 at 108 days.

Fig 7.9 Response (thymidine incorporation) of foetal efferent lymphocytes to Con A.

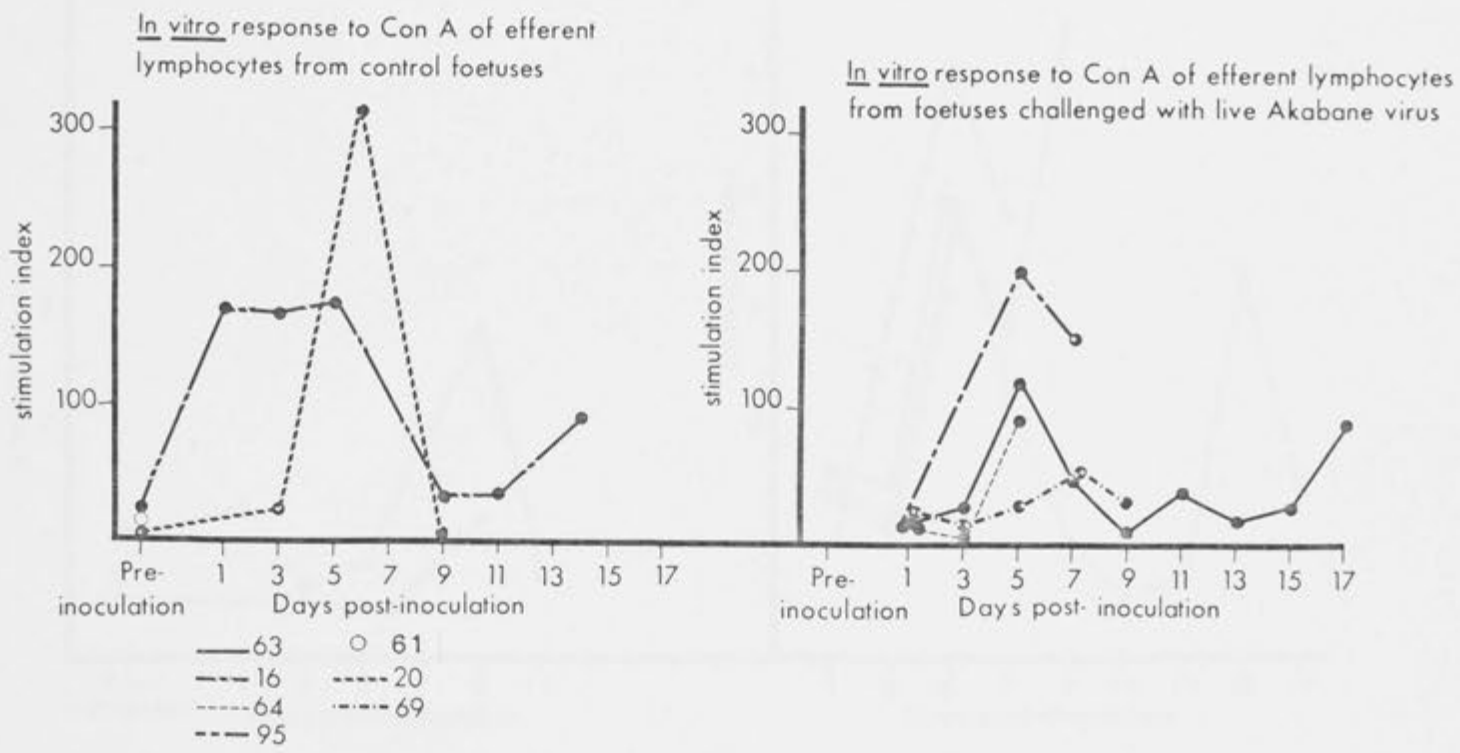


Fig. 7.10. Response (Thymidine incorporation) of foetal efferent lymphocytes to Akabane virus.

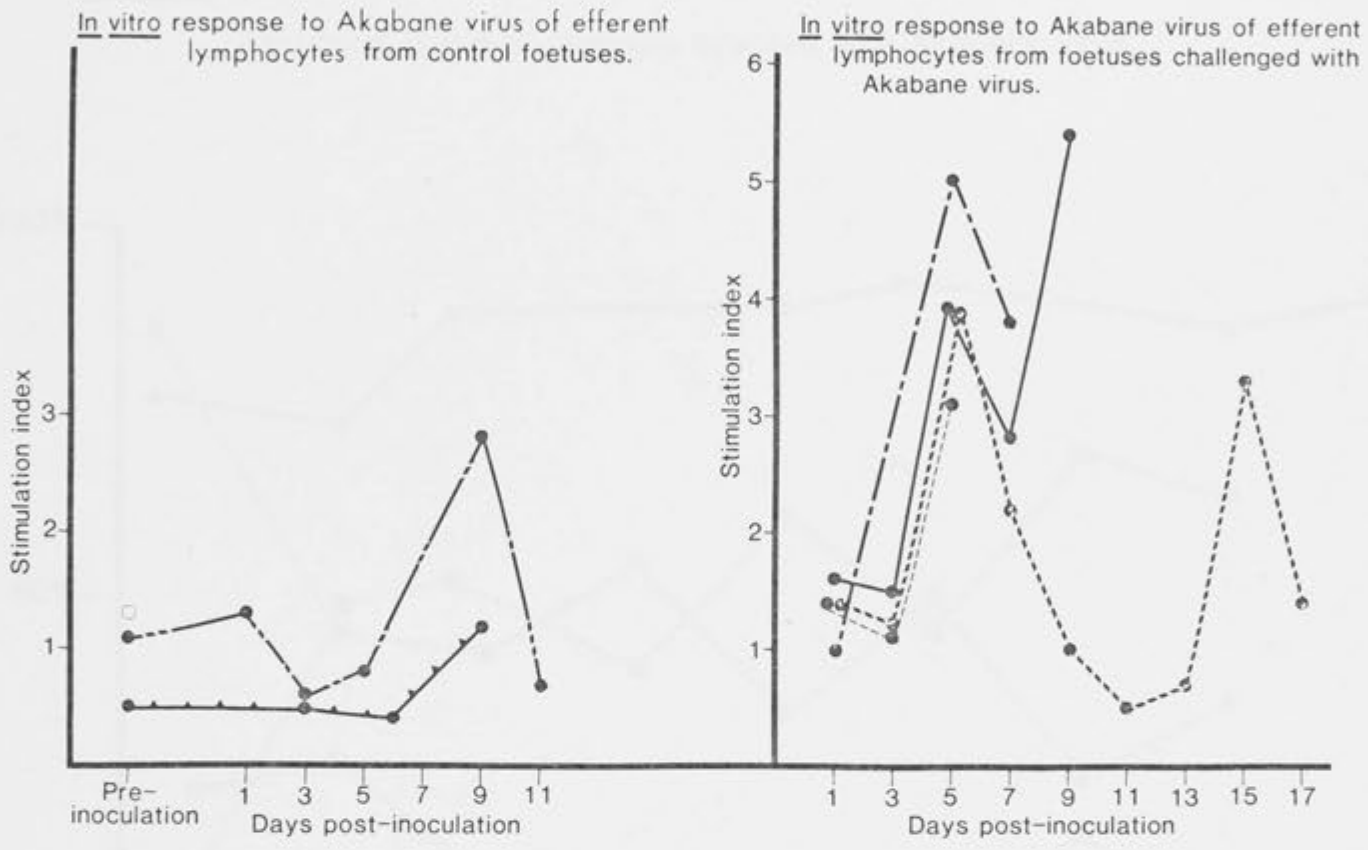
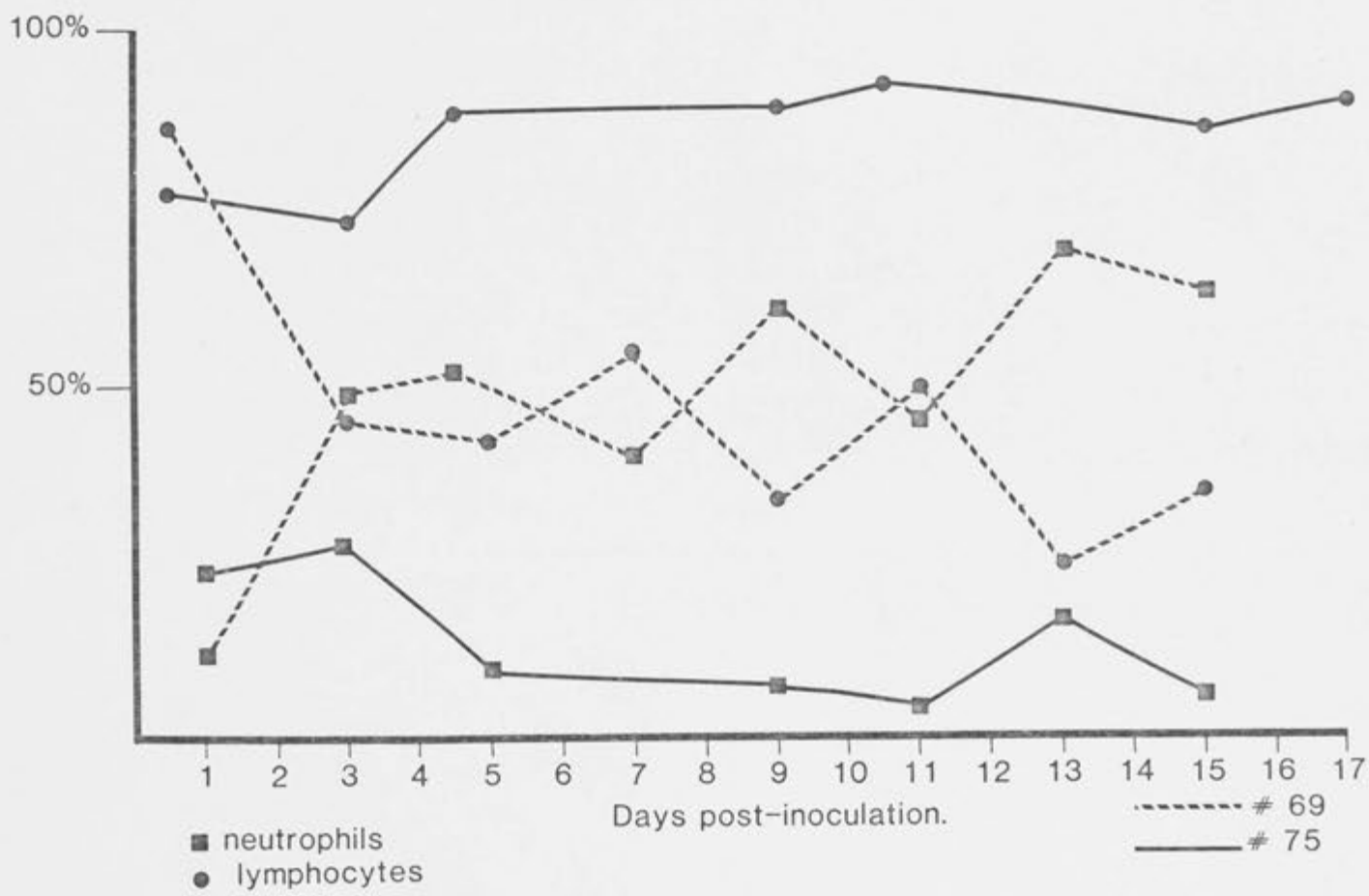


Fig 7.11 Relative proportions of lymphocytes and neutrophils in peripheral blood of foetuses infected with Akabane virus.



Lymphocytes were drained from foetus # 69 but not from # 75.

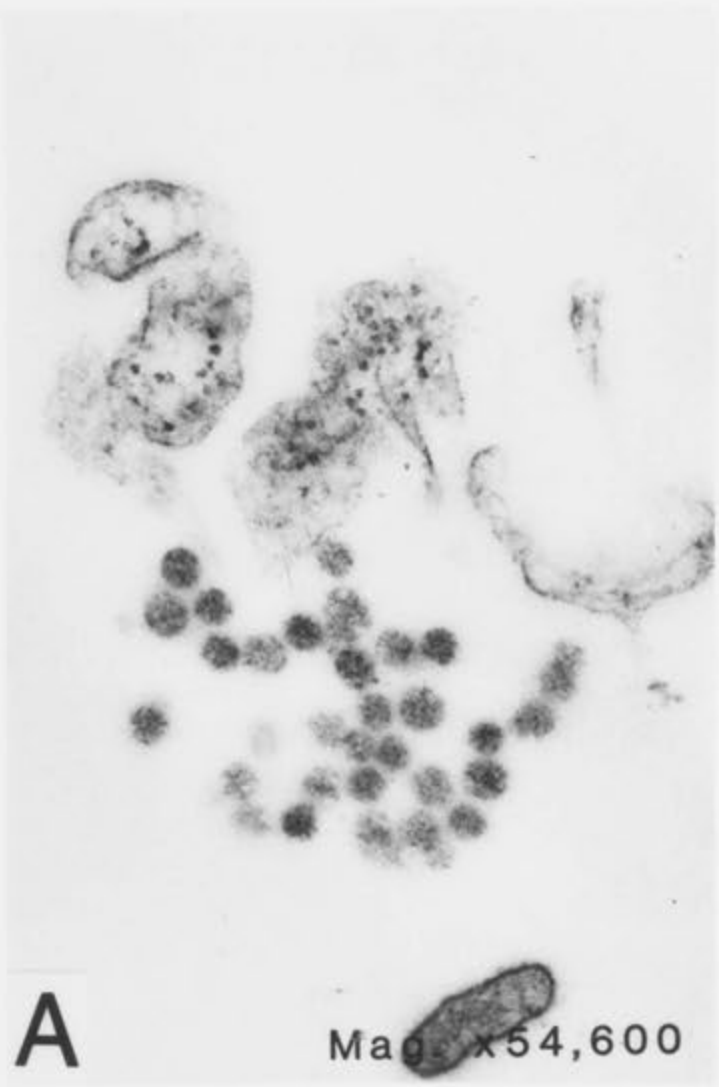
PLATE 7.3 Electron micrographs of cultured Akabane virus.

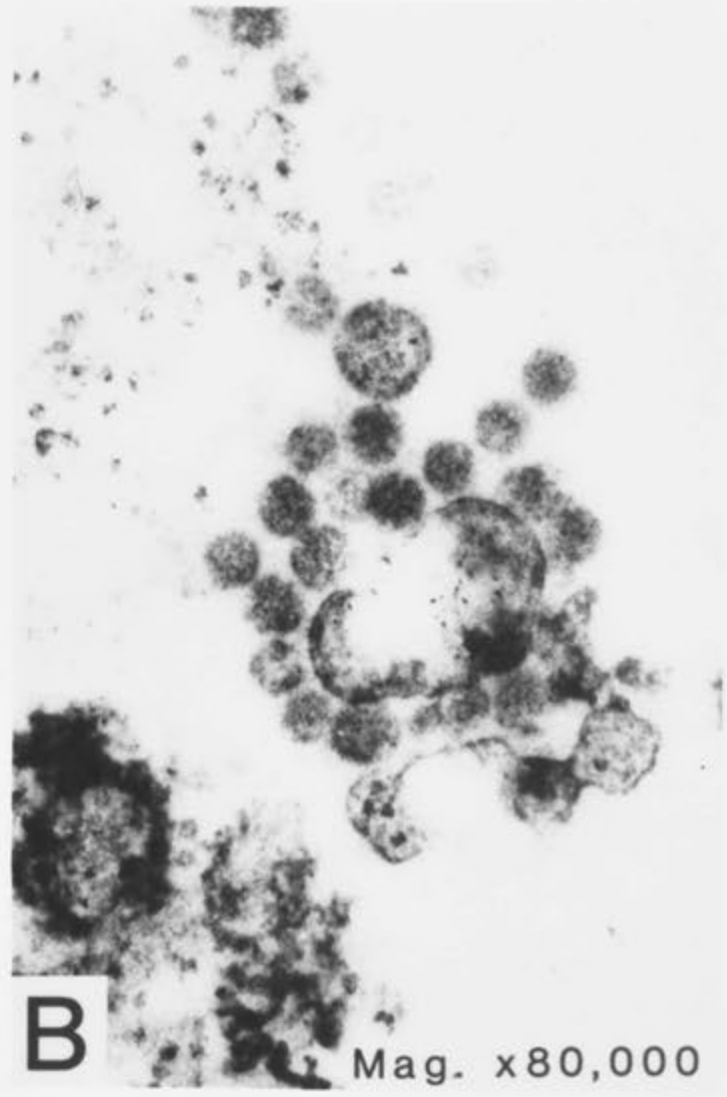
A: Akabane virions purified by ultrafiltration and set in agar. The virions are round, with an average diameter of 80 nm.

B: Akabane virions purified by ultrafiltration and set in agar. The virions are round, with an average diameter of 86 nm.

C: Akabane virions in the cytoplasm of cultured Vero cells, 24 h after addition of the virus to the culture.

D: Akabane virions in the cytoplasm of cultured Vero cells, 3 d after addition of the virus to the culture.


A
Mag. x54,600


B
Mag. x80,000


C
Mag. x54,600

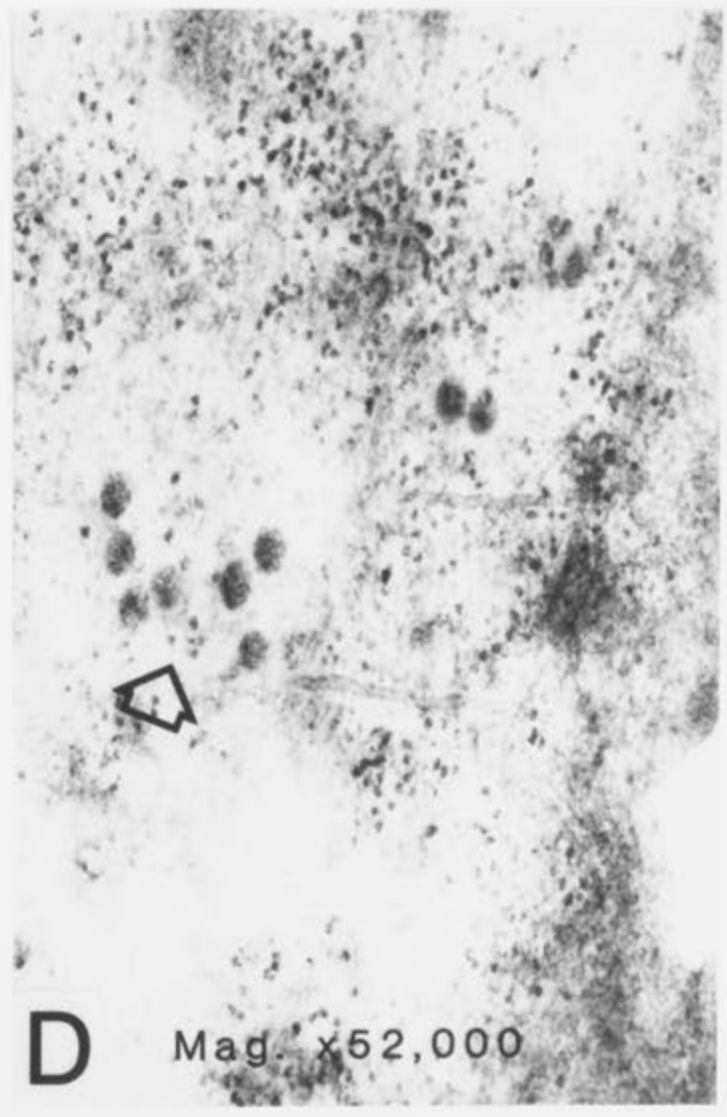
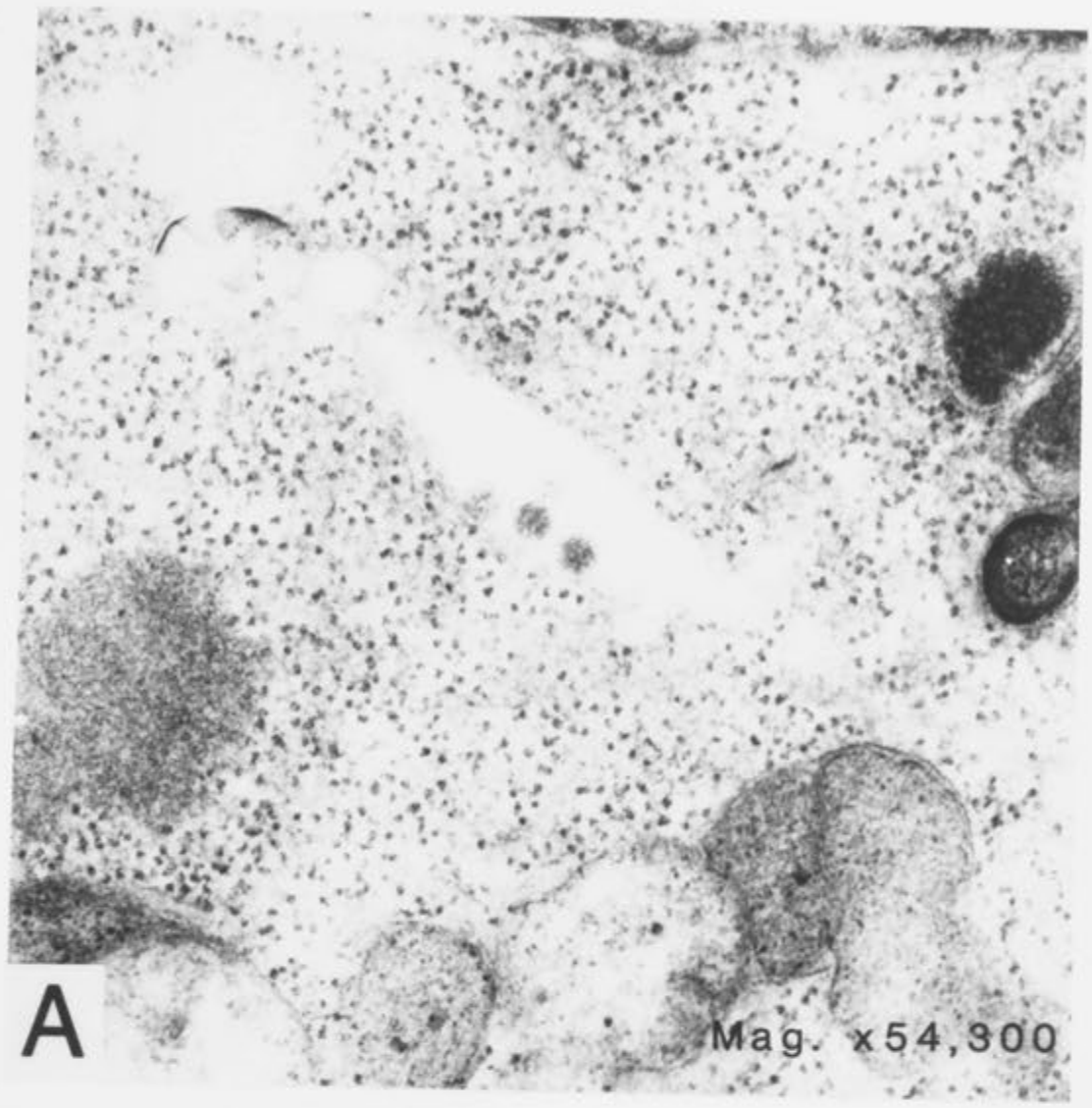

D
Mag. x52,000

PLATE 7.4 Electron micrographs of Akabane virus in efferent foetal lymphocytes infected *in vivo*.

A: Akabane virions in the cytoplasm of efferent lymphocytes 5d after challenge of the local lymph node with viable Akabane virus at 104d gestation. The virions appear to be within a cytoplasmic vesicle.

B: A group of Akabane virions (arrowed) in the cytoplasm of efferent lymphocytes 5d after challenge of the local lymph node with viable Akabane virus at 104d gestation. The virions appear to be loose in the cytoplasm. The average diameter of these virions is 90 nm.


A
Mag. x54,300

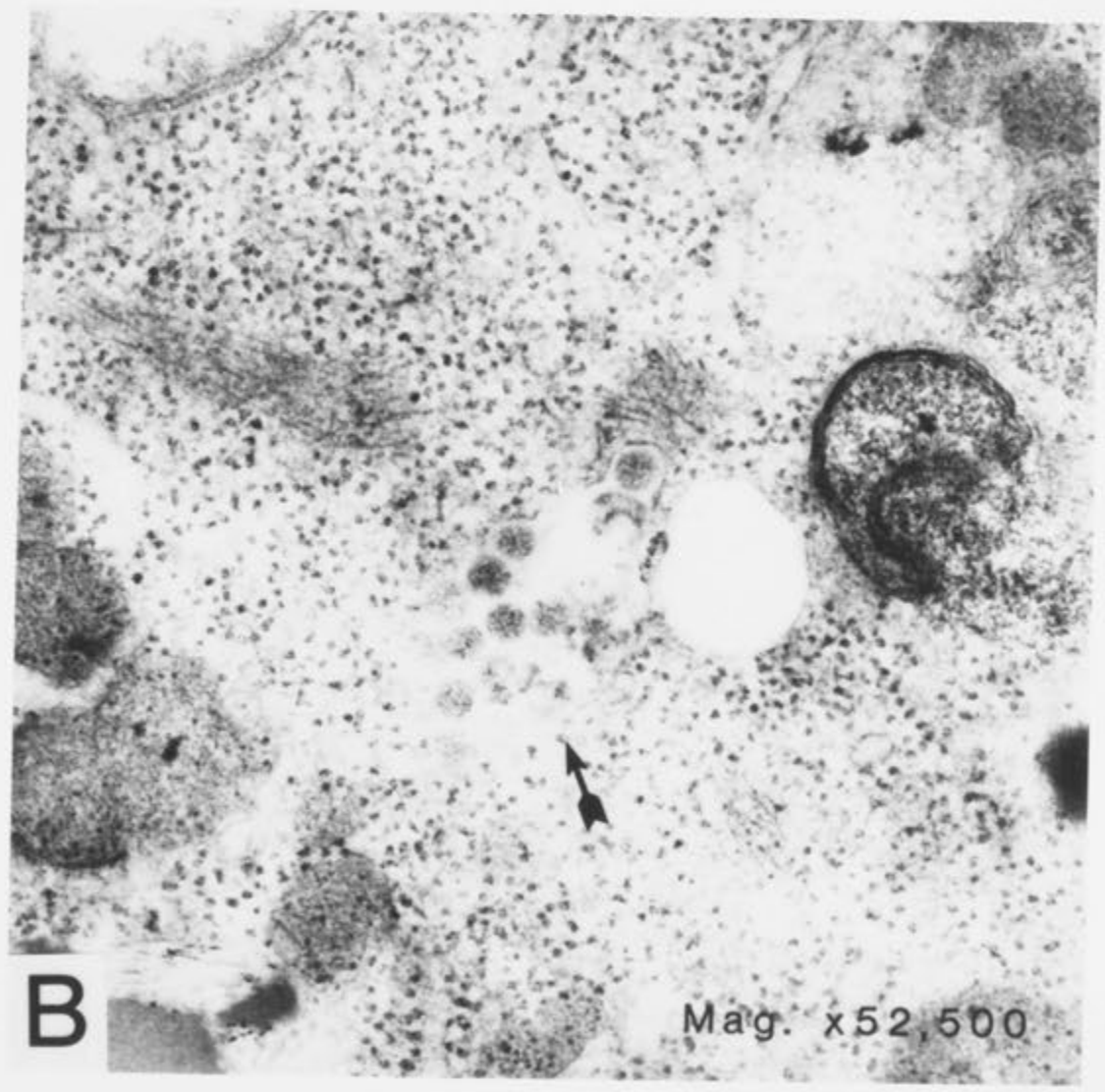
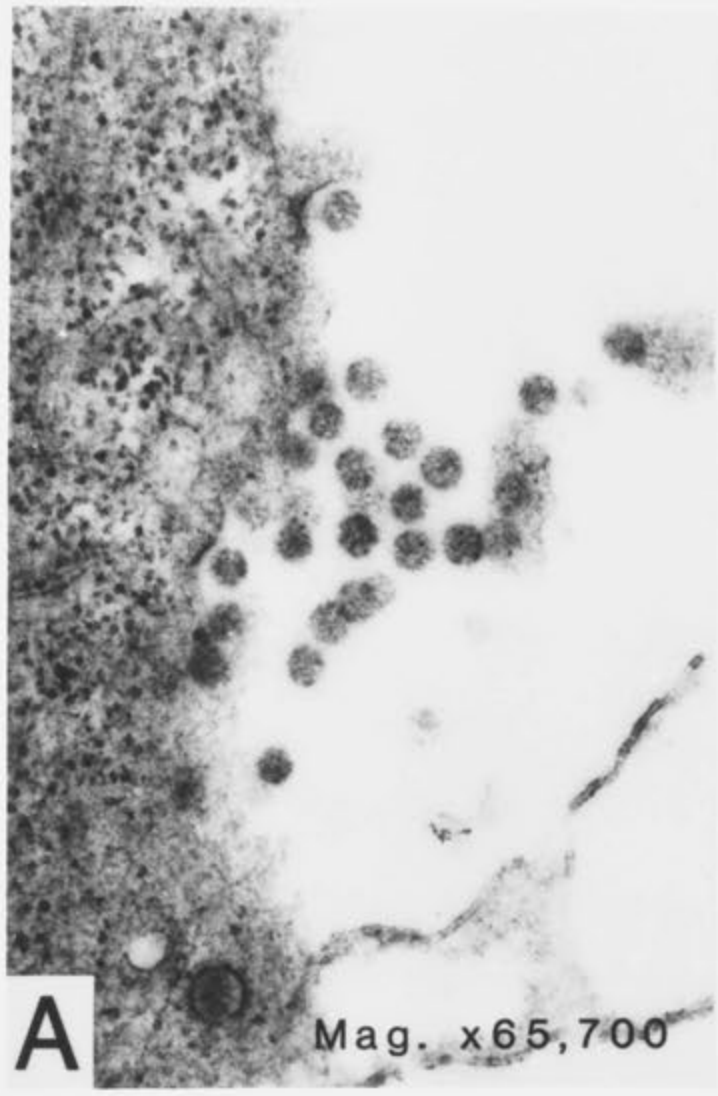

B
Mag. x52,500

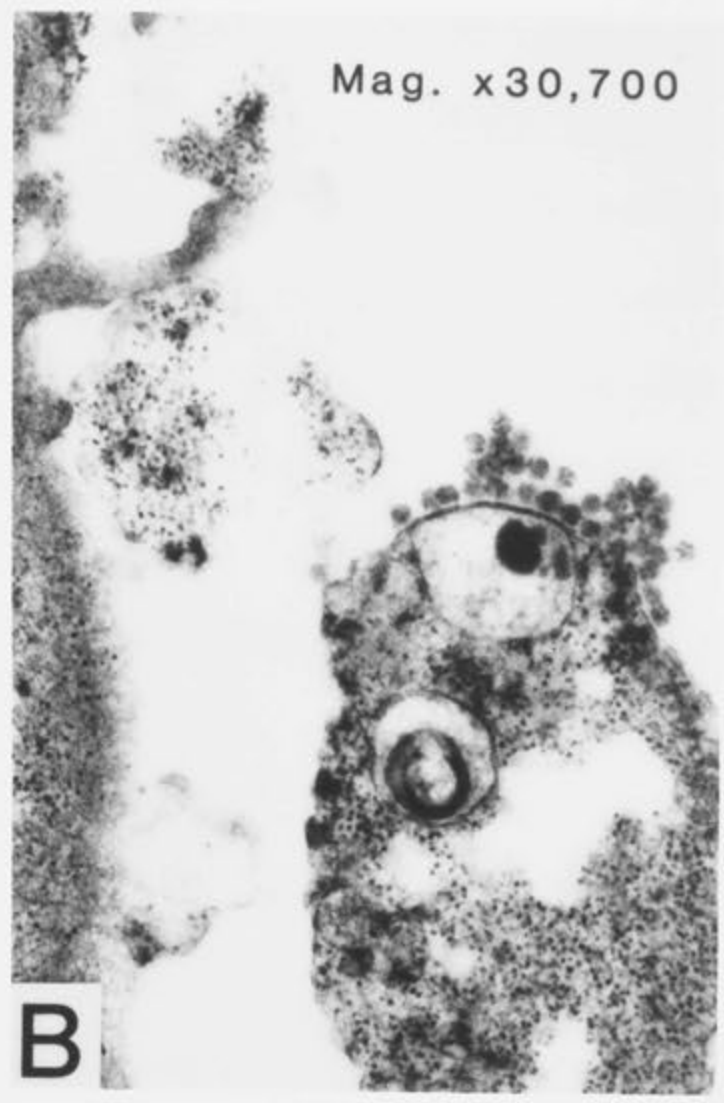
PLATE 7.5 Electron micrographs of Akabane virus in efferent foetal lymphocytes infected *in vivo* and *in vitro*.

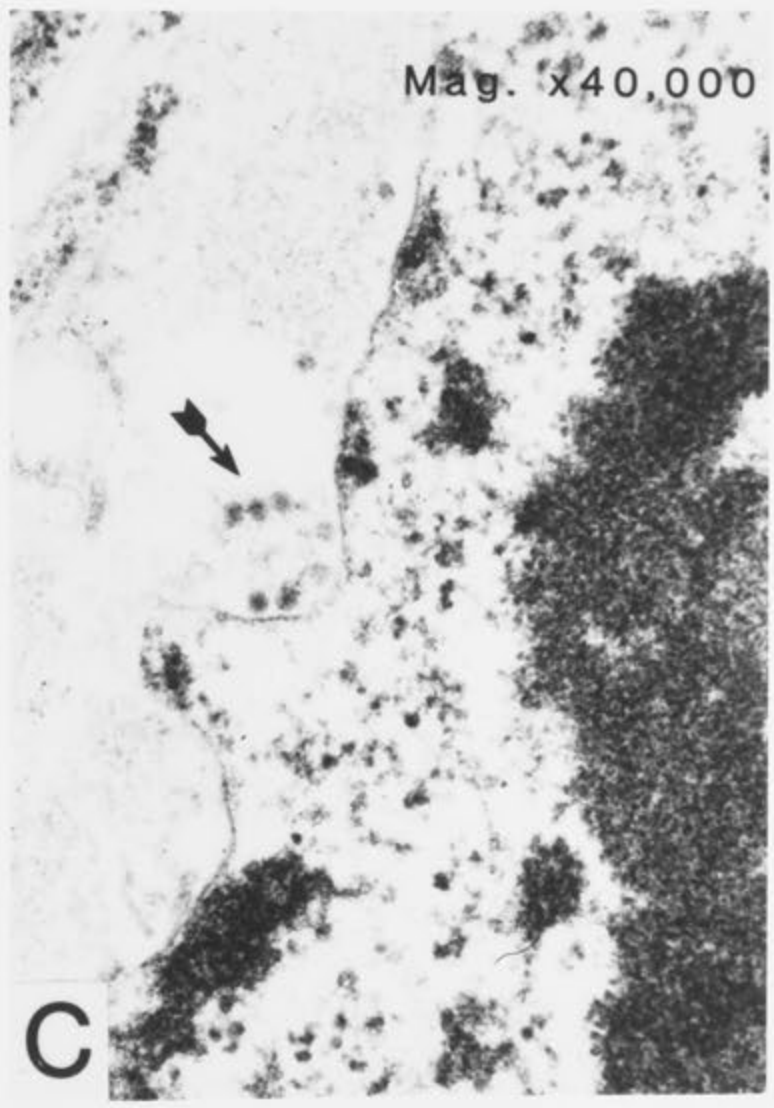
A: Akabane virions within and outside a foetal efferent lymphocyte at 109d gestation, 5d after challenge of the local lymph node with viable Akabane virus.

B: Extracellular Akabane virions, associated with a washed lymphocyte from a 109d foetus, 5d after challenge of the local lymph node with viable Akabane virus.

C: Akabane virions (arrowed) in the cytoplasm of a cultured foetal lymphocyte 4d after the addition of viable Akabane virus to the culture. The virions appear to be loose in the cytoplasm.


A
Mag. x65,700


Mag. x30,700
B


Mag. x40,000
C

search for Akabane virus in the foetal lymphocytes.

The purified Akabane virus grown and used for inoculations (Plate 7.3, A and B), morphologically resembled the virus as described by Ito et al, (1979). The virions were usually spherical, but sometimes oval, enveloped particles of average diameter 85 - 90 nm. Plate 7.3, C and D, shows these particles in Vero cell monolayers in which they were grown up. Particles morphologically similar to the Akabane virus injected into the foetuses were present extracellularly and in the cytoplasm of efferent lymphocytes collected from one infected foetus (#69) at one, five and eight days after local challenge with viable virus (Plates 7.4 and 7.5, A and B). The intracellular virions were usually less electron-dense than were extracellular virions, and were found in cytoplasmic spaces and rarely in membrane-bound vesicles. Only small numbers of virus particles were observed in either Vero cells or lymphocytes. Infected lymphocytes showed mild lesions of the cytoplasm, consisting of aggregation and rarefaction of cytoplasmic ground substance, resulting in a 'moth-eaten' appearance. Cell lysis was rarely observed. Infected cells were larger than uninfected cells, and were more frequently found at the top of the cell pellet implying an association between viral infection and large lymphocytes of low density. Virions were not detected in lymphocytes from control foetuses, nor in the lymphocytes from the remaining three animals injected with Akabane virus, although characteristic cytoplasmic abnormalities were present in lymphocytes of two of these.

Following the detection of Akabane virions within lympho-

cytes, the capacity of the virus to infect, survive and/or replicate in foetal lymphocytes *in vitro* was investigated. Efferent lymphocytes from two control foetuses (133 and 136 days of gestation) were cultured *in vitro* in the presence of a known titre of viable Akabane virus. Cells were harvested after two, four and six days culture, and examined for virus presence by electron microscopy and for virus titre by plaque assay.

Electron microscopic examination of four-day cultures of these lymphocytes failed to reveal virus in cultures to which it had not been added. Intracellular and extracellular Akabane virions were, however, present in lymphocyte cultures which had been infected (Plate 7.5). The site and appearance of these intracellular virions were consistent with those of *in vivo* intralymphocyte virions (Plates 7.4 and 7.5). The viral titres of *in vitro* cultures of efferent lymphocytes from the two uninfected foetuses, at intervals following addition of Akabane virus to the cultures and in the absence of added virus, are shown in Table 7.2. The titre dropped sharply over the six days of culture, but appreciable levels of viable virus were still present by six days after infection.

7.4 DISCUSSION

The type and severity of lesions observed in these S/C inoculated foetuses of 121 - 147 days gestation resembled those of foetuses infected intraperitoneally on days 50 - 120 However, only two of the four showed abnormalities. The oldest foetuses infected (at 123 and 126 days) had no lesions. Whilst this may have been a reflection of foetal maturity at

this age these two animals differed from the others in another important aspect. For several days following local S/C infection, all efferent lymphocytes from that draining node were removed, thereby greatly reducing the amount of virus available for dissemination throughout the tissues of the animal, the amount of intralymphocytic virus and presumably also the number of infected and stimulated and/or primed lymphocytes. One or more of these factors may have modified the subsequent course of the disease. Presumably some viral antigen dissemination occurred in these two foetuses via the blood vascular system, bypassing the lymphoid system, as sensitization of lymphocytes from the unchallenged lymph node was observed (see Table 7.16, foetuses #63^{+} and 64$^{+)}$. Since the virus should therefore have had access to its target tissues where replication should be possible, drainage of lymph may not greatly reduce the quantities of virus passing to target organs. Lymph drainage would, however, reduce the availability of infected or primed lymphocytes, and this availability may be the chief influence on the severity of the virally induced lesions.

No evidence was found to support any explanation for the extensive necrosis of foetal and maternal placental tissue in #95, as the ewe had developed detectable serum levels of specific antibody by seven days after foetal inoculation. It is possible that contamination by another infectious agent may have been responsible. Necrosis was not a common feature of placentomes from pregnant ewes infected with Akabane virus (see Chapter 5), although mild placental lesions were seen in the directly inoculated foetuses of Chapter Six. This difference in the placental morphology between the foetuses of

Chapter Five and those of Chapters Six and Seven may be an effect of the age of the placenta at the time it is exposed to the virus (31 to 44 days as opposed to 50 to 126 days). Alternatively, it may be due to the nature of the source of the infection, although this seems to be less likely in view of the fact that viral titres in placentae were similar regardless of whether the virus was injected into the ewe or the foetus. Perhaps there was a reduction in potency of virus that has been exposed to maternal tissues in comparison with virus that has encountered only foetal tissues. Such a reduction would be due to non-specific factors as these ewes lacked specific antibody and cellular reactivity before inoculation, and were presumably not previously exposed.

The cerebellum is only beginning to expand rapidly and to differentiate late in gestation. Cerebellar lesions were first detected in a 65-day foetus (Table 6.3), but were of irregular occurrence even in the older foetuses. The lesions (loss of Purkinje cells and decreased cell content of the granular layer) resembled those described for other viral diseases causing cerebellar damage in response to infection in late gestation, such as feline panleukopænia virus (Kilham et al., 1967). Infection at this time has been shown to destroy the rapidly dividing cells of the external germinal layer, thereby preventing their migration and differentiation.

The presence of Akabane virus in lymphocytes of 108-126 day foetuses resulted in only mild morphological abnormality in these cells. There was no suggestion that widespread lysis of these efferent lymphocytes had occurred. It seems probable

that infected lymphocytes could effectively transport and distribute such virions throughout the body.

The sequential studies of lymphocytes draining from the prescapular efferent duct showed that challenge with viable virus was responsible for an increase in the proportion of cells bearing surface immunoglobulin. This increase apparently consisted entirely of cells bearing IgM, as is the case in an adult early primary response (Taussig, 1979). In each case, this increase immediately preceded the appearance of circulating antibody. Inactive viral antigen induced a smaller and later rise, which was not however followed by the appearance of circulating antibody. Presumably the antigen was recognized but it was insufficiently stimulating to result in a detectable response, just as it induced no detectable cell-mediated response. It appears that proliferation of the virus in the host is necessary in order for the virus to stimulate a good primary immune-response, either by producing sufficient antigen or by directly affecting the lymphocytes concerned in the response. Inactive viral antigen may however have been sufficient to prime for a secondary type immune-response. Administration of Vero antigen alone did not change the proportion of cells possessing surface-bound immunoglobulin. This is in contrast with the capacity of Vero antigen to stimulate lymphocytes (probably T cells) which then recognized it as an antigen <u>in vitro</u> (7.3).

Akabane virus challenge to the prescapular lymph node exerted little if any influence on efferent lymph flow rate. It induced a slight and transient increase in total cell out-

put, accompanied by a rise in the proportion of these cells which were lymphoblasts (Figs. 7.4 - 7.7), suggesting that the virus was activating lymphocytes *in vivo*. That sensitization of lymphocytes was occurring in the node was established with the demonstration of a specific response to Akabane antigen of these efferent lymphocytes *in vitro* (Table 7.16). Pre-treatment with Akabane virus resulted in a non-significant decrease in *in vitro* response to Con A of foetal lymphocytes. In contrast, it resulted in a highly significant ($p < 0.001$) increase in the *in vitro* response to Akabane virus (see Table 7.17). This indicates that the response to Akabane virus was probably specific. Sensitization reached a maximum about five days after inoculation, thus coinciding with the peak in the cells bearing surface immunoglobulin, and preceding the appearance of circulating antibody. The virus did not itself act as a mitogen, since it did not stimulate unprimed cells. Neither inactivated viral antigen nor Vero antigen had any effect on specific responses (Fig. 7.10).

Inoculation of the foetus with inactivated viral antigen, Vero antigen and viable Akabane virus each amplified non-specific *in vitro* lymphocyte responses, but the amplification due to viable virus tended to be smaller than that due to either control inoculum (Fig. 7.9). It is possible that Akabane infection depressed the non-specific responsiveness of the foetal lymphocytes. Local challenge of adult lymph nodes with Akabane virus also resulted in a transient rise in the lymphoblast output by the node, and efferent lymphocytes from two of these three ewes gave specific if inconsistent *in vitro* responses to Akabane virus. The only difference between the

TABLE 7.17 - Statistical information derived from Tables 7.15 and 7.16

Pretreatment	Response (SI) at 5 Days	
	Con A	Viable Akabane virus
+AV	n = 6	n = 6
	$\bar{x}$ = 97.38	$\bar{x}$ = 3.53
	SD = 54.39	SD = 0.86
-AV	n = 3	n = 3
	$\bar{x}$ = 167	$\bar{x}$ = 0.83
	SD = 120.89	SD = 0.37

This table compares the response at five days after inoculation to Con A and to Akabane virus of lymphocytes from Akabane virus-treated foetuses (+AV) and from untreated foetuses (-AV). Pretreatment with Akabane virus resulted in a non-significant decrease in response to Con A, and a highly significant ($p<0.001$) increase in response to Akabane virus.

foetal and adult results was the greater consistency of the foetal results, and this may have been due to improved technical proficiency in performing the *in vitro* assays as the foetal experiments were done after the adult experiments.

The foetus from which data on lymphocyte output were obtained for longer than nine days (#63), showed a biphasic response in both humoral and cell-mediated immune responses. The response of lymphocytes to specific and non-specific activators *in vitro* exhibited a second peak at approximately 15 days after inoculation (Figs. 7.9 and 7.10), coinciding with a further rise in the blast cell output of the node (Fig. 7.6). There was also a second rise in circulating antibody titre (Fig. 7.8), closely followed by a second rise in the percentage of cells with surface bound immunoglobulin (Table 7.14). In all cases the second peak was smaller than the first.

In any electron microscopic study there is potential for considerable sampling error. The error was reduced so far as possible by sectioning at three levels of the blocks, and by examination of 200 cells per sample. Large numbers of virions were never seen in lymphocytes. This was also the case when Vero cells that had been cultured in the presence of virus were examined by electron microscopy. This suggests that a high rate/degree of replication of Akabane virus is not necessary for the production of cellular lesions.

These results show that Akabane virus is taken up by lymphocytes. The virus appears to survive in the lymphocytes, as there was no change in virion morphology after eight days,

and it is possible that they may have replicated there. The presence in the cytoplasm of lymphocytes of lesions apparently caused by the virus supports this hypothesis. Alternatively, the increase in intracellular virion numbers may have been a scavenging effect although lymphocytes do not usually have a phagocytic function.

The in vivo findings of Akabane virions within lymphocyte cytoplasm were confirmed by the in vitro studies. Not only did infection of lymphocytes occur, but it is possible that there was also viral replication in these lymphocytes. The fall in titre over the duration of culture indicates that long-term persistence of virus in cells under these culture conditions would not have occurred. However, Akabane virus has a short half-life in cell-free medium at high temperatures, approximately four hours at 37°C (Della-Porta, personal communication) and would not be detectable after six days at 37°C unless either protection by the cells permitted its survival or replication occurred.

The cultured lymphocytes were not subcultured, nor was their medium renewed during the six days. It is thus not known whether a decline in cell function and/or viability was responsible for the falling viral titre, or whether the virus was not capable of long-term persistence in lymphocytes in vitro.

It is possible that the production of only minor cytopathic effects may facilitate wider dissemination of lymphocytes bearing Akabane virus before the infected cells and virions are phagocytosed. It may also be relevant to the in-

terpretation of those observations to note that the survival and possible replication of virus was observed in foetal lymphocytes, and these cells came from a lymph node involved in an immune response as indicated by its rising output of lymphoblasts. On both counts these cells would have been expected to differ in function from the normal adult lymphocyte population. Conceivably, differences in functional capacity, of foetal compared with normal adult lymphocytes, may represent a similarity with the transformed or neoplastic lymphocytes that have been reported to sustain replication of other viruses.

It was not technically feasible to identify the relationship of lymphocytes containing virus to lymphocytes producing antibody, so it is not known whether there is a correlation between intra-lymphocyte virus and antibody production.

The role of the lymphoid system is usually considered to be one of protection against infections (Berger et al, 1981). However, in view of the detection of Akabane virus in the cytoplasm of efferent lymphocytes, it is important to consider the role of the lymphoid system in the pathogenesis of the disease. The draining lymph node is of great potential importance in limiting an infection. Provided it can limit the spread of a virus, the target tissues (in the case of Akabane, the central nervous system (Hartley et al, 1977)), will not be exposed to direct attack. If, however, the lymphocytes and macrophages take up the virus and provide an environment suitable for its replication, they represent a very rapid and thorough means of viral dissemination throughout the body.

Some viruses may replicate in lymphoid cells. Lymphoid structures have been claimed to be the primary site of Poliovirus multiplication, and to form an important pathway for subsequent viral spread to target organs such as central nervous tissue (Bodian, 1955). Transmission of some morbilliviruses in lymphocytes during viraemia has been reported (Fraser and Martin, 1978; Summers et al, 1978, 1979). Bovine virus diarrhoea antigen has been detected by immunofluorescence in mononuclear cells, mainly macrophages (Ohmann, 1983), and the mononuclear phagocyte system is considered to be essential for the spread of this infection to the target intestinal tissues. Reisang (1973) suggested that hog cholera virus may spread via the lymphoid system to its principal target, intestinal tissue.

Although there are no reports of viral replication in foetal lymphocytes, there has been some work done on the capacity of adult lymphocytes from various species to support viral replication.

Several viruses have been detected by electron microscopy within lymphocytes from animals infected in vivo. These include Rubella virus in a patient with rubella-associated arthritis (Chantler, Ford and Tingle, 1981), and virus particles similar to leukotic 'C' type viruses in lymphocytes from cattle that were serologically positive for leukosis virus (Mateva and Feodorov, 1981).

Viruses have been detected more frequently in cultured lymphocytes from diseased animals. In some instances, the viruses have been the causative agents in non-neoplastic

diseases present in the animal. Examples are bovine virus diarrhoea virus which was detected in cultured lymphocytes from calves with mucosal disease (Ohmann, 1982), and Papovavirus which was detected in cultured lymphocytes from patients with concurrent measles infections (Lecatsas, Schoub, Rabson and Joffe, 1976). In other cases, viruses have been demonstrated in cultured lymphocytes taken from animals with neoplastic diseases. Included in this category are Herpesvirus (Giddons, 1975; D'aichenko, Kakubava, Lapin, Iakovleve and Beriia, 1976; Campbell and Woode, 1970), and leukovirus (Karpas, Wreghitt and Nagington, 1978). It has also been shown that continuous lymphoblastoid cell lines can be produced by infection of normal cultures with some neoplastic viruses, such as murine leukaemia viruses (Rosenberg, Baltimore and Scher, 1975), and Epstein-Barr virus (Stoerker, Yajima and Glaser, 1982).

In addition to their presence in lymphocytes cultured from diseased animals, viruses have been demonstrated in lymphocytes taken from normal animals, and activated in culture by phytohaemagglutinin, lipopolysaccharide or allogeneic cells. These transformed cells supported replication of viruses with which they were infected including vesicular stomatitis virus (McFadden, Truitt and Shechmeister, 1973), and Herpesvirus (Bouronche, Clausen and Darner, 1970; Bruns, 1980; Teute, Braun and Kirschner, 1984). A continuous lymphoblastoid cell line was shown to permit replication of tick-borne encephalitis virus and Venezuelan equine encephalomyelitis virus (Melik-Andreasian, Barinski, Guschin, Skorikove and Labzo, 1981).

Thus the presence of virus in lymphocytes often appears to be associated with clinical diseases usually considered to be a result of defects in cell-mediated immunity, such as concurrent viral diseases and leukaemias. It has also been reported in association with transformation or activation of cells and with viruses causing malignant or persistent diseases. However, in this study Akabane virus was present within the cytoplasm of lymphocytes from infected foetuses, despite its non-neoplastic, non-persistent nature.

In conclusion, the foetus in late pregnancy is susceptible to direct infection and virally induced tissue damage. It is likely that the inflammatory component of the lesions is contributed to by sensitized lymphocytes. Circulating foetal lymphocytes can support the survival and probably the replication of Akabane virus. The foetus infected in late gestation can mount specific cellular and humoral responses to the infection, which resemble the responses seen in adult ewes.

DISCUSSION

DISCUSSION

The study of the development of immunocompetence by the developing embryo and foetus requires the introduction of various antigens into the embryo and foetus *in utero* or in culture *in vitro*, and/or to the components of the reticulo-endothelial system cultured *in vitro*. Many antigens which are suitable for use in the adult are lethal when introduced into the embryo or foetus. Accordingly, in these experiments the chosen antigens for the study of development of immunogenesis were those of sheep lymphocytes, antigens most likely to be encountered by the sheep foetus, and a virus which is non-pathogenic in the adult but which is transmitted to the foetus where it causes lesions which are usually non fatal.

After attempts to culture the sheep embryo *in vitro* and to use alloantigens *in vivo* proved unsuccessful, attention was directed to Akabane virus antigens for use in the adult ewe, newborn lamb, sheep foetus and cultured adult and foetal lymphocytes.

Three discrete factors could influence the infection of the foetal lamb with Akabane virus, namely the accessibility of the foetus to the virus, the susceptibility of foetal target organs and the competence of the foetal immune mechanisms over a wide range of foetal ages.

While foetal responses observed in this experimental system may be relevant to the naturally occurring situation, extrapolation to naturally occurring foetal infection would be subject to some constraints. These arise because of

possible differences in the mode of viral entry to the foetus, the infecting dose delivered and the presence of additional components in the inocula, all of which may modify the host response.

Access of the foetus to Akabane virus

Accessibility of the foetus to virus involves two components, the first being the ability of the virus to cross the placenta and the relative facility of such transmission at different stages of gestation. Previous work has demonstrated that injection of the CSIRO 16 strain of Akabane virus into the pregnant ewe will infect foetuses aged 32 to 36 days and cause foetal lesions (Parsonson et al, 1981b). Work with other strains has shown transplacental infection between 28 and 101 days (Hashiguchi et al, 1979), but foetal lesions were seen only in those infected between 29 and 81 days (Narita et al, 1979). The present investigation demonstrated that Akabane virus is capable of producing disease in ovine foetuses infected between 31 days and 120 days of gestation and that this capacity is certainly lost by the time of birth. Direct inoculation of the foetus resulted in infection of placentome and transmission to the ewe. Maternal inoculation was followed by the establishment of a focus of infection in the placenta where the virus replicated, and thence by infection of the foetus, probably by release of infectious progeny virus into the foetal circulation. Mims (1968) considered that, while the placenta is a physical barrier to the free passage of viral particles, the junction remains a real barrier to viral transmission only if the virus is unable to replicate in these cells.

In the present investigation, failure to demonstrate Akabane viral transmission between 18 and 22 days of gestation may indicate that natural infection of the foetus does not occur in the absence of a placenta, as Emerson and Delez (1965) found with the hog cholera in pigs. Requirement for placentation could be due to a requirement for close contact between placental cells or for provision of a site for viral replication.

However, foetuses were susceptible to both infection and disease after direct inoculation of the foetus with Akabane virus between 50 and 120 days. This suggests that despite the absence of gross abnormalities in previous studies, foetal disease may also occur after maternal infection later than 91 days gestation. Although transplacental infection of the foetus later in gestation was not attempted in this study, the presence of specific maternal antibody following direct foetal infection between 50 and 120 days presumably indicated trans-placental transmission of the virus from the foetus. This suggests that any loss of foetal susceptibility to Akabane infection and disease later in pregnancy (Parsonson et al, 1977; Hashiguchi et al, 1979) is of foetal origin. However, the placenta in later gestation may effectively reduce the dose of passaged virus below that required to produce disease. For example, the placental haemodynamics which have been suggested to be a factor in foetal infection (Sellers, 1969) may vary with maturation. The ability of ovine foetal tissues to produce interferon does not appear to have been studied. The human placenta manufactures interferon in response to Rubella virus infection and, conceivably, similar activity on

the part of the sheep placenta could be of importance in the transmission of Akabane virus. Given the diversity of experimental designs employed in Akabane virus investigations, it is possible that variation in the ability of different strains of the virus to cross the placenta could account for some of the apparent contradictions.

In addition to the relative facility of transmission of the virus across the placenta at different ages and therefore the dose of virus received, the pattern of transmission may also be important. Since the virus replicated similarly in placenta regardless of the source of infection, the total dose delivered to the foetus is likely also to have been similar. While this argues against a dose effect to explain the differences between directly and indirectly infected foetuses, the kinetics of virus delivery rather than the dosage might be the significant influence. The transplacentally delivered dose may have been lower for some time before peak placental replication was reached, resulting in a continuous slow release rather than a sudden discrete pulse. Such a variation in the pattern of access of the virus might affect the pattern of infection of the foetal organs, or the nature of the foetal immune response. For example, prolonged exposure to low doses may tend to induce tolerance rather than immunity.

Susceptibility of target organs and pathogenesis

The second factor which could influence the outcome of infection is the susceptibility of the foetal target organs. This susceptibility may depend upon the maturity of the foetus and thus on the stage of differentiation attained by

the tissue. Alternatively, it may depend on access of the virus to the target tissue. There may be barriers of some kind (e.g. the blood-brain barrier) preventing viral access to some tissues or at some stages of foetal maturation. It may be that the virus enters target tissues only when within cells, and that entry of such carrier cells varies with tissues and/or with foetal age. A third possibility is that, perhaps due to surface receptors, the virus can infect some tissues only, regardless of their stage of development.

The distribution of virus in infected foetuses was similar, regardless of age at infection or route of inoculation, the virus showing tropism for placenta, CNS, skeletal muscle and lymphocytes. This tropism may be due to rapid division and differentiation of the target cells, the presence or concentration of cell surface antigens or receptors enabling viral entry, or to metabolic factors such as rate, O_2 or CO_2 levels, or nutrient supply.

Except in the case of cerebellum, foetal age did not appear to influence target organ susceptibility. Cerebellar lesions were found in infected foetuses of 69-125 days of age but not in younger foetuses and susceptibility of this organ may have depended on maturation having reached the stage of Purkinje cell differentiation, since these were the cells most obviously affected. However, the influence of maturation on target organ susceptibility might have become important at birth, after which infection failed to cause lesions.

Akabane virus localized, replicated and persisted in the placenta, particularly in the foetal trophoblast. Persistence

in the placenta occurred despite the production of both maternal and specific foetal antibody. This may be because the antibody does not have access to trophoblast cells. Alternatively, as suggested by Parsonson et al. (1981a), the concentration of antibody may not have been sufficiently high to be effective. A further possibility is that, like many other viruses (Berger et al, 1981) elimination of Akabane virus, at least from this site, is dependent on cellular rather than humoral mechanisms. Since the same cell types, identified by fluorescent microscopy to be trophoblast and syncytial cells, were involved at all ages, it appears that the virus prefers this cell type for its growth. McPhee (personal communication) has found that Akabane virus will replicate and persist in cultured placental cells, while the closely related Aino virus will not. Surface receptors or some metabolic function of these cells may favour growth of the virus. Mechanisms such as the preferential metabolite erythritol which is believed to encourage the growth of Brucella abortus in the placenta (Smith et al, 1962) are less likely to be involved as the virus replication occurs intracellularly and should depend only on the normal nutrition of the host cell. Akabane virus did not appear to be severely cytopathic for trophoblast even allowing for a possibly high rate of regeneration.

Except for the placenta of #95, the proportion of cells which were necrotic was very small compared with the number showing presence of viral antigen. Similarly, in other congenital viral infections virus has been recovered from placentomes in which no microscopic changes were found (Kendrick, Schneider and Straub, (1971).

Virus was also detected in lymphocytes but rarely in blood cells or plasma. In some viral infections, such as rinderpest (Tajima and Ushijima, 1971), lymphoid tissues are damaged by the virus, but central lymphoid tissues did not appear to be infected or damaged by Akabane virus. In the case of the cannulated foetuses #63 and #64, virus was detected in sites other than the infected node, despite the removal of all their efferent lymph. It thus appears from these studies that the virus spreads from the various sites of infection by haematogenous as well as lymphatic routes, probably as free virions and as intracytoplasmic particles in lymphocytes of those foetuses old enough to possess them. There have been reports of transmission of other viruses (measles and canine distemper) in lymphocytes during viraemia (Summers, Greisen and Appel, 1978). There was no evidence from electron or fluorescent microscopical results during this study that the virus survived intracellularly in phagocytic cells, unlike BVDV in calves which does so in macrophages (Ohmann, 1983). This ability to infect, survive and apparently to replicate in circulating lymphocytes without causing rapid cell lysis is particularly important in that it provides a means of rapid distribution to target tissues while protected from specific and non-specific immunological attack. Infection of lymphocytes by viruses other than morbilliviruses usually occurs only when the virus is oncogenic or when the cells have been transformed. Although in the absence of information on whether lymphocytes of adult sheep also carry Akabane virus no conclusions can be drawn, the immaturity of the foetal lymphocytes may be responsible.

In view of the tropism of Akabane virus for both CNS and lymphocytes, it is interesting to note that Reif and Allen (1964) found lymphocytes and CNS cells of mice to share some surface receptors. If infection of cells by Akabane virus is receptor-dependent, trophoblast and skeletal muscle cells are likely to share the relevant receptor with CNS cells and lymphocytes.

The mechanisms of viral entry to target tissues are entirely speculative. Virus may enter the CNS via infection of endothelium, as indicated by the detection of antigen in endothelial cytoplasm, but this does not exclude other routes, such as direct passive movement across capillary walls, infection of choroid plexus and ependyma, or carriage via infected leukocytes when cellular infiltration occurs. Invasion of the brain may be enhanced by factors such as haemodynamic changes as reported by Sellers (1969) for a number of viruses. Similar routes of entry could be suggested for infection of skeletal muscle.

The development of Akabane disease in this study was assessed on the basis of gross and histological morphology at intervals after infection. It is probable that the deformities would develop further by the end of gestation and that the full extent of the nervous lesions would not be apparent until muscles and joints had had time to develop.

Some understanding of the mechanisms by which Akabane virus induces lesions has been gained from this study. Since the virus did not cause pyrexia in the ewe, foetal CNS abnormalities were not attributable to maternal hyperthermia as

were those observed by Hartley, Alexander and Edwards (1974) in lambs from ewes exposed to high temperatures. However, the effect, if any, of the virus upon foetal body temperature is unknown.

Interruption of placental function is unlikely to be important since little histological damage was observed, and regeneration of trophoblast if it did occur appeared to be effective. Nor was virus recovered from all tested placentomes of an infected placenta. This observation was unexpected since it seemed unlikely that placentomes would vary in their resistance to viral infection. The most likely explanation is that the interval between maternal infection and placental examination was too short for all the placentomes to have been comparably infected. However it is also possible that the variation in the degree of placentomal development and/or vascularization resulted in differences in susceptibility. Alternatively, all placentomes may have been infected but to differing degrees, and the amount of virus present in some may have been insufficient to be detected by the assay used. However, despite the lack of evidence of morphological abnormalities, it is possible that foetal deformities were aggravated by interference with a basic metabolic function, either of the foetus proper or more likely of the placenta. For example, interference with transport or metabolism of copper could result in the congenital focal cerebral degeneration observed in cases of maternal copper deficiency (Everson, Schrader and Wang, 1968).

The distribution of the virus in foetal tissues favoured

a hypothesis of occurrence of direct cytopathic effect as an explanation for the damage produced. The distribution of viable virus and viral antigen correlated positively with the pathological changes, and there was no sign of thrombosis or infarction. With one exception, no host cellular response was observed in foetuses infected earlier than 44 days, thus inflammation did not contribute to the necrosis in these foetuses. A similar basis has been put forward as the basis of bluetongue virus pathogenesis after Osburn et al, (1971b) used fluorescent labelling of viral antigen to demonstrate that the viral cytopathic effect resulted directly in hydranencephaly of foetal lambs. As virus, viral antigen and histological lesions were all detected in skeletal muscle, it is likely that the skeletal muscle lesions and arthrogryposis observed in newborn and aborted ruminants were at least partly due to direct viral damage to this tissue, rather than being solely attributable to secondary neurogenic atropny as previously suggested (Hartley et al, 1977). Direct muscular damage has been demonstrated to be the basis for the posterior paralysis in mice infected with Ross River virus (Murphy, Taylor, Mims and Marshall, 1973).

While the same tissues were involved in all infected foetuses, in older foetuses, directly inoculated between 50 and 109 days, a host inflammatory response was superimposed on the primary viral damage, being most severe when infection occurred in the 50-80 day range. In view of the greatly increased severity of necrosis and disturbance of tissue architecture associated with lesions involving this inflammatory infiltrate, the inflammation is unlikely to be just an

irrelevant outcome of interaction between virus and tissue. Thus in addition to the malformations and abnormalities caused by viral infection of cells, the foetal immunological response to this infection also contributed to the lesions by causing cellular infiltration and tissue necrosis. This enhanced the tissue damage but did not extend the range of tissues involved. With one exception, this infiltration consisted entirely of mononuclear cells - some monocytes but chiefly small and large lymphocytes. The lack of neutrophil infiltration suggests that complement was not involved.

It is not possible to say how specific was this infiltration. Opinions on the specificity of other virally induced mononuclear infiltrations in brain vary. Enright and Osburn (1979) considered that that occurring in bluetongue virus infection of sheep was non-specific. On the other hand, the inflammatory response in Sindbis virus encephalitis of adult mice has been shown to be immunologically specific, since after its ablation, reconstitution required the inoculation of virus-specific sensitized lymphoid cells (McFarland, Griffin and Johnson, 1972). If the inflammation present in Akabane disease were antigenically specific, it would imply that the virus induces a cytotoxic T cell and/or delayed hypersensitivity response by the host.

In the study using cannulated foetuses, described in Chapter Seven, the two foetuses from which all efferent lymphocytes from a challenged node were removed developed no histopathology, despite the presence of virus in CNS. The two foetuses whose efferent lymphocytes from the infected

node were not removed developed inflammatory lesions of the CNS. The drainage of efferent lymphocytes was shown to decrease the circulating lymphocyte population of both peripheral blood and lymph, and thus the total number of available lymphocytes. This suggests that a possible reason for the lack of inflammatory lesions is that insufficient lymphocytes were present in circulation. It may be that it was the number of circulating sensitized lymphocytes that was important, rather than the total circulating number. This would suggest that the infiltration depended on specifically sensitized lymphocytes, implying the presence of a specific cellular immune response.

However, whether specific or non-specific in nature, the fact that younger foetuses did not mount such an inflammatory response suggests that it reflects a developing immunocompetence. Since the direct infection of foetuses younger than 50 days resulted in their death, it is not known whether the age at infection or the route of infection was responsible, but in view of the inflammation present in the only foetus infected transplacentally after 40 days gestation (only one such infection was attempted), the age at infection is more likely to be the relevant factor. It appears from the reports of Border disease in foetal sheep (Barlow and Patterson, 1982; Barlow, 1983) that the outcome of infection with BVDV similarly varies with foetal age at infection because of the stage of immune development, since infection after the development of specific immune competence at approximately 80 days resulted in inflammatory tissue damage which did not usually occur after earlier infection. Those cases in which it did were

attributed to precocious onset of immunocompetence. Similarly, the inflammatory response to bluetongue virus observed by Osburn et al. (1971b) in CNS of foetal sheep infected after 75 days may have been immunologically mediated. Inflammation tended to be less severe in foetuses infected after 80 days, for which increased immunological maturity was probably responsible. Onset of the humoral response is likely to have been earlier, resulting in clearance of virus before severe inflammation developed.

This hypothesis of pathogenesis of Akabane infection provides an explanation for the observations of Kirkland (personal communication) and Narita et al. (1979) of a variation in lesions with the age of the foetus at infection, for example the hydranencephaly of foetuses infected early would be due to gradual necrosis over a long period, while the poliomyelitis and encephalitis of those infected later in gestation would be due to immune cell involvement with its associated inflammatory-induced tissue destruction.

Host immunocompetence

The third factor likely to influence the outcome of foetal infection with Akabane virus is the competence of the host's immune defence mechanisms. Two aspects of this competence are of relevance here. The different immune mechanisms are likely to mature at different rates and would thus be expected to vary with age. Secondly, some of these immunological mechanisms are likely to be more effective than others in the protective immunity against Akabane viral infection.

Interpretation of the immune responses by the foetal lamb

requires consideration of the mature ovine response to Akabane virus and of the maturation of general immune responsiveness of the foetus. Infection of adult ewes resulted in a specific humoral response, sometimes very rapid in onset suggesting secondary characteristics. Since these animals were seronegative for neutralizing antibody against Akabane virus before infection, this rapid humoral response may be due to previous exposure to cross-reacting antigens. Alternatively, it might be the normal primary response to Akabane of a mature ewe. Local challenge of lymph nodes with viable virus resulted in a local cellular immune response as shown by efferent cellular characteristics and specific *in vitro* activity, and systemic challenge also sensitized the cellular immune system. Knowledge of the response of the immunocompetent host is thus available for comparison with that of the foetus.

While transplacentally infected foetuses produced no detectable neutralizing antibody to Akabane virus, they may have done so if left beyond the 75 days at which the oldest was autopsied. Specific antibody has been found by other workers (Hashiguchi *et al*, 1979; Parsonson *et al*, 1981a) in foetal lambs of 64 and 75 days from ewes infected with Akabane virus at 32 to 36 days. Nor was antibody found in foetuses infected directly, I/P or S/C, and recovered before 75 days of age. However, ten of the 11 foetuses autopsied at 75 days or later had developed antibody by the time they were recovered, confirming previous work with this virus (Parsonson *et al*, 1981a). This antibody appeared considerably earlier than did antibodies to *Brucella ovis* and bluetongue virus (Osburn and Hoskins, 1969; Osburn *et al*, 1971a). The exception was a

foetus recovered nine days after inoculation. It appears from this and from the time of antibody appearance in lymph from infected nodes that the capacity to mount a detectable specific humoral immune response to Akabane did not develop until approximately 75 days, irrespective of the duration of exposure to the virus. Once they had developed this capacity, foetuses required approximately ten days after exposure to produce detectable antibody titres. Since inactivated virus did not induce antibody formation, virus infection of cells and/or virus replication seems necessary to supply adequate stimulation of humoral immunity in the foetus in the absence of artificial enhancers of antigenicity such as adjuvants. The presence and replication of the virus may have modified the developing host response and delayed its effective competence. This could be examined if Akabane-infected foetuses were challenged with non-replicating antigens for which the normal age of specific antibody appearance is known.

The foetuses infected transplacentally at 31-40 days showed no evidence of developing a specific humoral or cellular response to the virus before they were killed at up to 75 days. The only foetus infected transplacentally later than 40 days (44 days), while it did not develop a humoral response, did show a lymphoid cell response, namely a focal mononuclear cell infiltration associated with viral-infected cells. There is thus a suggestion that the capacity for a cellular type of response had been impaired by early exposure (31 to 40 days) to the virus. Since there is no information available on the *in vitro* cellular reactivity of 80 to 94 day foetuses after their transplacental infection at 31 to 40 days to compare

with that of the foetuses infected after 50 days, it is not possible to distinguish retarded acquisition of reactivity from non-acquisition. Nor is there evidence to indicate whether the suggested depression of cellular response was specific or non-specific. The failure to demonstrate specific antibody in the five oldest transplacentally infected foetuses (that is, three aged 75 days, one aged 73 days and one aged 71 days) when compared with the presence of antibody in the only directly infected 75 day foetus to be tested, suggests that the humoral immunological capacity might also have been impaired. However, the humoral response reported in 64 and 75 day foetuses after transplacental infection at 32 to 36 days (Hashiguchi *et al*, 1979; Parsonson *et al*, 1981a), does not support this suggestion, and since foetuses infected at 31 to 40 days were not examined later than 75 days, it is not possible to resolve this point. If humoral suppression did not occur, cellular impairment may still have occurred in its absence.

Akabane viral challenge of the foetal prescapular lymph node induced sensitization of lymphocytes within that node (Chapter 7). Such sensitization reached a maximum about five days after inoculation, thus coinciding with the peak in the cells bearing surface immunoglobulin, and preceding the appearance of circulating antibody. In contrast, similar challenge of the adult ewe was less consistent in its induction of sensitization of the lymphocytes. However the consistent sensitization resulting from systemic challenge of ewes reached a maximum at five to nine days after challenge immediately preceding the peak specific neutralizing antibody

response.

The viability of the virus was an important factor in the induction of a response by the adult and foetal hosts. Injection of inactivated Akabane virus did not result in lesions, specific neutralizing antibody or specific *in vitro* cellular response. Other workers have examined this question using other viruses. Rosenberg, Farber and Notkins (1972) studying lymphocyte transformation after Herpes simplex infections of rabbits, found better stimulation of sensitized spleen cells with inactivated viral antigen, while Griffin and Johnson (1973) who worked with Sindbis virus infection of mice found that the live virus was more effective than inactivated virus in stimulating lymphocytes *in vitro*. Griffin *et al*, suggested that this difference may have been due to the fact that herpes, unlike Sindbis, replicates in mononuclear cells *in vivo* and *in vitro* thereby depressing DNA synthesis. Apparently in the case of Akabane virus, the intralymphocyte presence of viable virus does not cause detectable depression of DNA synthesis. Since Kirkland (personal communication) induced specific humoral responses in adult cattle with inactivated Akabane virus plus adjuvant, one would expect the inactivated preparation used in this study to possess the antigenic determinants necessary to stimulate the immune system. Perhaps the replication of the virus is necessary to provide sufficient antigen to induce a detectable response or to enable antigen presentation by macrophages. Alternatively, there may be a non-specific adjuvant effect from live virus that augments the specific response to viral antigen. If this were the case, inactivated Akabane virus plus another

live virus might induce the production of anti-Akabane virus antibody.

Lambs infected on the day of birth with viable virus all mounted a specific humoral response of primary character, but gave no evidence of cellular immune response or inflammatory lesions. These findings were in contrast with those for similar challenge of foetuses, which developed local cellular immune responses and severe inflammatory lesions. That is, there was no evidence of a cellular immune response by the newborn, despite the elimination of the virus from these infected lambs. This observations casts doubt on the importance of the cellular response in the protective immune response to primary infection, and implies that humoral immunity alone may be sufficient to eliminate the virus and prevent disease.

It has been widely suggested that antibody alone is not sufficient to clear viruses from infected hosts, and that a cell-mediated immune response is necessary for virus elimination by eliminating virus-infected cells (Stott and Osburn, 1980; Berger and Blanden, 1981; Banatvala, 1977), but evidence for this is lacking. The contrary conclusion could well be drawn from these studies, in which the humoral immune system appeared to be important in the elimination of Akabane virus.

Relative importance of cellular and humoral immunity

For several reasons, the humoral immunological response rather than the cellular response appears to be chiefly responsible for terminating Akabane viral infection of foetuses. Firstly, a lymphoid cellular response was apparent from as

early as 58 days gestation in foetuses infected intraperitoneally, while large titres of virus remained recoverable from these foetuses up to the age of 89 days. Presumably the lymphoid cells involved did not achieve cytolysis of all infected cells before they released infectious progeny virus.

It is possible that the viral capacity to survive intracellularly in lymphocytes may specifically or non-specifically reduce the effectiveness of these cells in terminating the infection, or that the development of immunocompetence involves differential maturation of different T cell functions.

The second reason is the suggestion, raised by the apparent depression of *in vitro* response to Con A by infected adults and viraemic foetuses, that the virus may impair at least the non-specific component of the cellular response.

Thirdly, there was a consistently positive correlation in infected neonatal and foetal lambs between the presence of specific neutralizing antibody and the absence of viable virus, despite the lack of demonstrable specific cellular reactivity in all bar one of these animals. The youngest foetus (75 days) to produce detectable levels of virus-specific antibody was also the first foetus to have eliminated viable virus from its non-placental tissues.

Although specific antibody was only detected in directly inoculated (I/P or S/C) foetuses of 75 days and older, the decrease in viral titre, particularly in brain, with passage of time after inoculation also occurred in much younger animals. While specific antibody appears to be necessary for

the elimination of viable virus, it is likely that some other factor is responsible for a decrease in the rate of viral replication. Such a factor might be the availability of only a limited number of susceptible host cells, or a non-specific immunological response.

The hypothesis is thus that the foetus is incapable of eliminating Akabane virus before it becomes able to produce specific antibody, at approximately 75 days. Such antibody then clears the virus from infected tissues by inactivating the virions released from the necrotic cells. Specific cell-mediated immunity may become increasingly important in viral clearance from the older foetus and adult.

Possible immune suppression

While some of the results from infected adults and from foetuses infected after 40 days gestation suggested the possibility of viral suppression of specific and non-specific cellular responsiveness, the evidence for this was not conclusive. The question of a more general immunological suppression in foetuses infected earlier than 41 days also arises from the results of this study.

The ovine foetus infected with Akabane virus either transplacentally or directly from 44 days of gestation had apparently developed some cellular immunocompetence by 58 days, and specific humoral immunocompetence by 75 days. On the other hand, in contrast with reports of other studies, foetuses infected between 31 and 40 days had not produced specific humoral immune response by 75 days. Nor had they

mounted any cellular inflammatory response. While the absence of information on these foetuses beyond 75 days prevents any conclusions, this evidence suggests that this virus may retard or abolish the development of the immune response if exposure occurs before 44 days gestation, permitting persistence of the virus. Since other workers have found antibody to Akabane virus in foetal lambs infected at this age (Parsonson et al, 1981), such suppression may not always occur, or it may be reversed later in gestation. A somewhat similar phenomenon has been observed in BVDV infection of foetal lambs, where infection in the first half of pregnancy tends to result in viral persistence with no detectable immune response, while later infection is followed by humoral and cellular responses and elimination of the virus (Zakarian, Barlow and Rennie, 1975; Barlow and Patterson, 1982). In the case of this virus, persistence endures for some years (Ohmann et al, 1982b).

This persistence of Akabane virus in the absence of immune response, noted in the present study, suggested that further investigation into the possible development of tolerance in the foetal lamb may be profitable. While tolerance of antigens can be induced in newborn and even adult rodents, Nossal and Pike (1980) reported that the threshold for B cell tolerance induction in foetal mice was very much lower than for the newborn when the tolerogen was introduced two days before the first appearance of any B cells in the foetus. If sheep are similar, this suggests that a tolerogen would be most effective if introduced by approximately 35 days. Such

a time is consistent with the observed lack of immunological response by foetuses exposed to Akabane virus, and with reported failure to induce tolerance of alloantigens in foetal sheep by primary exposure between 45 and 110 days (Mitchel, 1959; Moore and Rowson, 1961; Miyasaka and McCullagh, 1982).

REFERENCES

REFERENCES

Amoroso, E.C. (1952) Placentation in Marshall's Physiology of Reproduction, Vol. II, 3rd Edn. Ed. Parkes, A.S. Longmans, Green and Co., London. Pp 127-297.

Andersen, A.A. and Campbell, C.H. (1978) Experimental placental transfer of Akabane virus in the hamster. American Journal of Veterinary Research 39, 301-304.

Anderson, D., Billingham, R.E., Lampkin, G.H. and Medawar, P.B. (1951) The use of skin grafting to distinguish between monocygotic and dizygotic twins in cattle. Heredity 5, 379-397.

Asantila, T. and Toivanen, P. (1976) Specificity of allogeneic and xenogeneic cell recognition in the fetal lamb. Journal of Immunology 117, 555-562.

Asma, G.E.M., Pichler, W., Schuit, H.R.E., Knapp, W. and Hijmans, W. (1977) The development of lymphocytes with T- or B-membrane determinants in the human foetus. Clinical and Experimental Immunology 29, 278-285.

Assheton, R. (1906) The morphology of the ungulate placenta, particularly the development of that organ in the sheep, and notes upon the placenta in the elephant and hyrax. Philosophical Transactions of the Royal Society B.198:143.

Bach, F. and Hirschhorn, K. (1964) Lymphocyte interaction: a potential histocompatability test in vitro. Science, 143, 813-814.

Banatvala, J.E. (1977) In Infections and Pregnancy, Ed. C.R. Coid, Academic Press, London. P. 454.

Banatvala, J.E., Potter, J.E. and Best, J.M. (1971) Interferon response to Sendai and Rubella viruses in human foetal cultures, leucocytes and placental cultures. Journal of General Virology 13, 193-201.

Barlow, R.M. (1980) Morphogenesis of hydranencephaly and other intracranial malformations in progeny of pregnant ewes infected with pestiviruses. Journal of Comparative Pathology 90, 87-98.

Barlow, R.M. (1983) Some interactions of virus and maternal/foetal immune mechanisms in Border disease of sheep. Progress in Brain Research 59, 255-268.

Barlow, R.M. and Gardiner, A.C. (1969) Experiments in Border disease. I. Transmission and pathology and some serological aspects of the experimental disease. Journal of Comparative Pathology 79, 397-405.

Barlow, R.M. and Patterson, D.S.P. (1982) Border disease of sheep: a virus-induced teratogenic disorder. IN Advances in Veterinary Medicine, Vol. 36. Verlag Paul Parey, Berlin.

Barlow, R.M. and Storey, I.J. (1977) Myelination of the ovine CNS with special reference to Border disease. I. Qualitative aspects. Neuropathology and Applied Neurobiology 3, 237-253.

Barlow, R.M., Vantsis, J.T., Gardiner, A.C., Rennie, J.C., Herring, J.A. and Scott, F.M.M. (1980) Mechanisms of natural transmission of Border disease. Journal of Comparative Pathology 90, 57-65.

Berger, M.L. and Blanden, R.V. (1981) The T lymphocyte in infectious pathology. Pathology Research Practice 171, 128-141.

Billingham, R.E., Brent, L. and Medawar, P.B. (1953) Actively acquired tolerance of foreign cells. Nature 172, 603-606.

Billingham, R.E., Brent, L. and Medawar, P.B. (1955) Acquired tolerance of tissue homografts. Annals of New York Academy of Science 59, 409-415.

Blood, D.C. (1956) Arthrogryposis and hydranencephaly in newborn calves, incidence and clinical findings. Australian Veterinary Journal 32, 125-131.

Bodian, D. (1955) Emerging concept of Poliomyelitis infection. Science 122, 105-108.

Boshier, D.P. (1969) A histological and histochemical examination of implantation and early placentome formation in sheep. Journal of Reproduction and Fertility 19, 51-61.

Bouroncle, B.A., Clausen, K.P. and Darner, E.M. (1970) Replication of Herpes simplex virus in cultures of Phytohemagglutinin-stimulated human lymphocytes. Journal National Cancer Institute 44(5), 1065-1078.

Bowen, R.A., Howard, T.H. and Pickett, B.W. (1982) Interaction of bluetongue virus with preimplantation embryos from mice and cattle. American Journal of Veterinary Science 43, 1907-1911.

Bruns, H. (1980) Electron microscopic studies of Herpes simplex virus Type 1 infection of macrophages, T- and B-lymphocytes of mice. Archives of Virology 64(3), 257-268.

Burnet, F.M., Stone, J.D. and Edney, M. (1950) The failure of antibody production in the chick embryo. Australian Journal of Experimental Biology and Medical Science 28, 291-297.

Buxton, A. (1954) Antibody production in avian embryos and young chicks. Journal of General Microbiology 10, 398-410.

Campbell, J.G. and Woode, G.N. (1970) Demonstration of a Herpes-type virus in short-term cultured blood lymphocytes associated with Marek's Disease. Journal of Medical Microbiology 3(3), 463-473.

Chantler, J.K., Ford, D.K. and Tingle, A.J. (1981) Rubella-associated arthritis: Rescue of Rubella virus from peripheral blood lymphocytes two years post-vaccination. Infection and Immunity 32(3), 1274-80.

Cinader, B. and Dubert, J.M. (1955) Acquired immune tolerance to human albumin and response to subsequent injections of diazo human albumin. British Journal of Experimental Pathology 36, 515-529.

Clarke, G.L. and Osburn, B.I. (1978) Transmissable congenital demyelinating encephalopathy of lambs. Veterinary Pathology 15, 68-82.

Clarkson, S.G., Doering, J.V. and Runner, M.N. (1969) Growth of post-implantation mouse embryos cultured in a serum-supplemented, chemically-defined medium. Teratology 2, 181-5.

Clawsol, A.B. (1928) Normal rectal temperatures of sheep. American Journal of Physiology 85, 251-270.

Cole, G.J. and Morris, B. (1971) The cellular and humoral response to antigens in lambs thymectomized in utero. Australian Journal of Experimental Biology and Medical Science 49, 55-73.

Colten, H.R., Silverstein, A.M., Borsos, T. and Rapp, H.J. (1968) Ontogeny of the first component of sheep complement. Immunology 15, 459-461.

Cybinski, D.H., St. George, T.D. and Paull, N.I. (1978) Antibodies to Akabane virus in Australia. Australian Veterinary Journal 54, 1-3.

D'Aichenko, A.G., Kakubava, V.V., Lapin, B.A., Iakovleva, L.A. and Beriia, L.I. (1976) Several molecular biologic characteristics of Herpes virus isolated from lympho-blastoid cell lines from Pavian hamadrils with malignant lymphoma. Votr. Onkol. 22, 81-85. Abstracted Medlars.

Davies, J. and Wimsatt, W.A. (1966) Observations on the fine structure of the sheep placenta. Acta anatomica (Basel) 65, 182-223.

Decker, J.M. and Sercarz, E.E. (1974) Early simultaneous appearance of antigen-binding cells in the foetal sheep. Nature 252, 416-418.

Della-Porta, A.J., Herniman, K.A.J. and Sellers, R.F. (1981) A serological comparison of the Australian isolate of Bluetongue virus type 20 (CSIRO 19) with Bluetongue group viruses. Veterinary Microbiology 6, 9-21.

Della-Porta, A.J., Murray, M.D. and Cybinski, D.H. (1976) Congenital bovine epizootic arthrogryposis and hydran-encephaly in Australia. Distribution of antibodies to Akabane virus in Australian cattle after the 1974 epizootic. Australian Veterinary Journal 52, 496-501.

Della-Porta, A.J., O'Halloran, M.L., Parsonson, I.M., Snowdon, W.A., Murray, M.D., Hartley, W.J. and Haughey, K.G. (1977) Akabane disease: isolation of the virus from naturally infected ovine foetuses. Australian Veterinary Journal 53, 51-52.

De Martini, J.C., Banks, K.L., Greenlee, A., Adams, D.S. and McGuire, T.C. (1983) Augmented T-lymphocyte responses and abnormal B-lymphocyte numbers in goats chronically infected with the retrovirus causing caprine arthritis-encephalitis. American Journal of Veterinary Research 44, 2064-2069.

Doherty, R.L. (1972) Arboviruses of Australia. Australian Veterinary Journal 48, 172-180.

Douglas, S.D. (1971) Human lymphocyte growth in vitro: morphologic, biochemical and immunologic significance. International Review of Experimental Pathology 10, 41-114.

Eidelzstein, P.R. and Ben-Sasson, S.Z. (1982) The role of non-specific lymphocytes in antigen-induced proliferative response in vitro. Journal of Immunological Methods 53, 27-39.

Emerson, J.L. and Delez, A.L. (1965) Cerebellar hypoplasia, hypomyelinogenesis, and congenital tremors of pigs, associated with prenatal hog cholera vaccination of sows. Journal of the American Veterinary Medical Association 147, 47-54.

Enright, F.M. and Osburn, B.I. (1979) Cytokine production by bluetongue virus-infected fetal sheep cells. Infection and Immunity 25, 403-407.

Enright, F.M. and Osburn, B.I. (1980) Ontogeny of host responses in ovine foetuses infected with bluetongue virus. American Journal of Veterinary Research 41, 224-229.

Everson, G.J., Schrader, R.E. and Wang, T.I. (1968) Clinical and morphological changes in the brains of copper-deficient guinea-pigs. Journal of Nutrition 96, 115-125.

Fahey, K.J. (1974) Immunological reactivity in the foetus and the structure of foetal lymphoid tissues. In Progress in Immunology II, Vol. 3 Ed. Brent, L. and Holborow, J. North-Holland, Amsterdam. Pp. 49-60.

Fahey, K.J. (1977) The response of foetal sheep to the somatic and flagellar antigens of Salmonella typhimurium. Australian Journal of Experimental Biology and Medical Science 55, 523-37.

Fahey, K.J. and Brandon, M.R. (1978) Synthesis of immunoglobulin by normal and antigenically stimulated foetal sheep. Research in Veterinary Science 25, 218-224.

Fahey, K.J. and Morris, B. (1978) Humoral immune responses in foetal sheep. Immunology 35, 651-661.

Fiscus, S.A., DeMartini, J.C. and Pearson, L.D. (1982) Mitogen-induced blastogenesis of peripheral blood and efferent lymph lymphocytes from sheep. American Journal of Veterinary Research 43, 629-632.

Ford, W.L. (1978) The preparation and labelling of lymphocytes. In Handbook of Experimental Immunology, Ed. Weir, D.M. Blackwell, Oxford. Pp. 23.1-23.22.

* Fuccillo, D.A. and Sever, J.L. (1973) Viral teratology. Bacteriological Reviews 37, 19-31.

Giddons, W.E. Jr. (1975) Replication of Herpes virus Saimiri in cultured lymphocytes of infected Owl Monkeys (Aotus trivirgatus). An electron microscopic and immunofluorescent study. Laboratory Investigation 32(4), 492-502.

Granberg, C. and Hirvonen, T. (1980) Cell-mediated lympholysis by foetal and neonatal lymphocytes in sheep and man. Cellular Immunology 51, 13-22.

* Fraser, K.B. and Martin, S.J. (1978) In Measles Virus and Its Biology Academic Press, London.

Green, W.W. and Winters, L.M. (1945) Prenatal development of the sheep. Minnesota Technical Bulletin 169, 2-35.

Griffin, D.E. and Johnson, R.T. (1973) Cellular immune response to viral infection: in vitro studies of lymphocytes from mice infected with Sindbis virus. Cellular Immunology 9, 426-434.

Guyer, M.F. (1953) In Animal Micrology, 5th edn. The University of Chicago Press. P.98.

Hall, J.G. and Morris, B. (1962) The output of cells in lymph from the popliteal node of sheep. Quarterly Journal of Experimental Physiology 47, 360-369.

Hall, J.G. and Morris, B. (1965) The immediate effect of antigens on the cell output of a lymph node. British Journal of Experimental Pathology 46, 450-454.

Hanan, R. and Oyama, J. (1954) Inhibition of antibody formation in mature rabbits by contact with antigen at early age. Journal of Immunology 73, 49-53.

Hartley, W.J. (1963) Selenium and ewe fertility. Proceedings of the New Zealand Society of Animal Production 23, 20-27.

Hartley, W.J., Alexander, G. and Edwards, M.J. (1974) Brain cavitation and micrencephaly in lambs exposed to prenatal hyperthermia. Teratology 9, 299-303.

Hartley, W.J., De Saram, W.G., Della-Porta, A.J., Snowdon, W.A. and Shepherd, N.C. (1977) Pathology of congenital bovine epizootic arthrogryposis and hydranencephaly and its relationship to Akabane virus. Australian Veterinary Journal 53, 319-325.

Hartley, W.J. and Haughey, K.G. (1974a) An outbreak of micrencephaly in lambs in New South Wales. Australian Veterinary Journal 50, 55-58.

Hartley, W.J. and Wanner, R.A. (1974b) Bovine congenital arthrogryposis in New South Wales. Australian Veterinary Journal 50, 185-188.

Hartley, W.J., Wanner, R.A., Della-Porta, A.J. and Snowdon, W.A. (1975) Serological evidence for the association of Akabane virus with epizootic bovine congenital arthrogryposis and hydranencephaly syndromes in New South Wales. Australian Veterinary Journal 51, 103-104.

Hashiguchi, Y., Nanba, K. and Kumagai, T. (1979) Congenital abnormalities in newborn lambs following Akabane virus infection in pregnant ewes. National Institute of Animal Health Quarterly 19, 1-11.

Hirsch, R.L., Griffin, D.E., Johnson, R.T., Cooper, S.J., de Soriano, I., Roedenbeck, S. and Vaisberg, A. (1984) Cellular immune responses during complicated and uncomplicated measles virus infections of man. Clinical Immunology and Immunopathology 31, 1-12.

Horstmann, D.M. (1969) Viral infections in pregnancy. Yale Journal of Biology and Medicine 42, 99-112.

Hotchin, J. and Weigand, H. (1961) Studies of lymphocytic chorio-meningitis in mice. I. The relationship between age at inoculation and outcome of infection. Journal of Immunology 86, 392-400.

Howard, J.G. and Mitchison, N.A. (1975) Immunological tolerance. Progress in Allergy 18, 43-96.

International Committee on Taxonomy of Viruses - fourth report (1982) Classification and nomenclature of viruses- the families and groups. Intervirology 17, 115-118.

Ito, Y., Kurogi, H., Takahshi, E., Goto, Y., Inaba, Y. and Omori, T. (1979) Electron microscopy of Akabane virus. Acta virologica 23, 198-202.

Jarrett, O. (1977) Prenatal viral infections and delayed post-natal disease. In Infections and Pregnancy. Ed. Coid, C.R. Academic Press, London. Pp. 515-550.

Janossy, G. and Greaves, M.F. (1971) Lymphocyte activation. I. Response of T and B lymphocytes to phytomitogens. Clinical and Experimental Immunology 9, 483-498.

Johnson, G.D., Holborrow, E.J. and Dorling, J. (1978) Immunofluorescence and immunoenzyme techniques. In Handbook of Experimental Immunology. Ed. Weir, D.M. Blackwell, Oxford. Pp. 15.1-15.30.

Johnson, K.P. and BYington, D.P. (1972) Hog cholera virus: multiple malformations produced by persistent, tolerant infection of fetal swine. Teratology 5, 259.

Johnson, K.P., Ferguson, L.C., Byington, D.P. and Redman, D.R. (1974) Multiple fetal malformations due to persistent viral infection. I. Abortion, intrauterine death, and gross abnormalities in fetal swine infected with hog cholera vaccine virus. Laboratory Investigation 30, 608-617.

Jordan, R.K. (1976) Development of sheep thymus in relation to *in utero* thymectomy experiments. European Journal of Immunology 6, 693-698.

Karpas, A., Wreghitt, T.G. and Nagington, J. (1978) Transformation of normal bone-marrow cells by a leukaemic cell line associated with a presumptive new human virus. Lancet 2(8098), 1016-1019.

Kendrick, J.W., Schneider, L. and Straub, O.C. (1971) Placental reaction to the infectious bovine rhinotracheitis-infectious pustular vulvovaginitis virus. American Journal of Veterinary Research 32, 1045-1051.

Kerr, W.R. and Robertson, M. (1954) Passively and actively acquired antibodies for Trichomonas foetus in very young calves. Journal of Hygiene, Cambridge 52, 253-263.

Kilham, L., Margolis, G. and Colby, E.D. (1967) Congenital infections of cat and ferrets by feline panleukopaenia virus manifested by cerebellar hypoplasia. Laboratory Investigations 17, 465-480.

Kurogi, H., Inaba, Y., Goto, Y., Miura, Y., Takahashi, E., Sato, K., Omori, T. and Matumoto, M. (1975) Serologic evidence for etiologic role of Akabane virus in epizootic abortion - arthrogryposis - hydranencephaly in cattle in Japan 1972-1974. Archives of Virology 47, 71-83.

Kurogi, H., Inaba, Y., Takahashi, E., Sato, K., Omori, T., Miura, Y., Goto, Y., Fujiwara, Y., Hatano, Y., Kodama, K., Fukuyama, S., Sasaki, N. and Matumoto, M. (1976) Epizootic congenital arthrogryposis - hydranencephaly syndrome in cattle: isolation of Akabane virus from affected foetuses. Archives of Virology 51, 67-74.

Kurogi, H., Inaba, Y., Takahashi, E., Sato, K., Satoda, K., Goto, Y., Omori, T. and Matumoto, M. (1977) Congenital abnormalities in newborn calves after inoculation of pregnant cows with Akabane virus. Infection and Immunity 17, 338-343.

Lascelles, A.K. and Morris, B. (1961) Surgical techniques for the collection of lymph from unanaesthetized sheep. Quarterly Journal of Experimental Physiology 46, 199-205.

Lecatsas, G., Schoub, B.D., Rabson, A.R. and Joffe, M. (1976) Papovavirus in human lymphocyte cultures. Lancet 2(7991), 907-8.

Leino, A. (1978) Ontogeny of PHA and Con A responses in the fetal lamb. Clinical Immunology and Immunopathology 11, 6-11.

Leino, A. and Soppi, E. (1979) Effect of thymosin on PHA and Con A responses by fetal lamb lymphocytes. Clinical Immunology and Immunopathology 13, 136-145.

Liggitt, H.D., DeMartini, J.C. and Pearson, L.D. (1982) Immunologic responses of the bovine foetus to parvovirus infection. American Journal of Veterinary Research 43, 1355-1359.

Lightbody, J., Bernoco, D., Miggiano, V. and Ceppellini, R. (1971) Cell-mediated lympholysis in man after sensitization of effector lymphocytes through mixed leukocyte cultures. Journal of Bacteriology, Virology and Immunology 64, 243-254.

McClure, T.J. and Dowell, A.E. (1968) Survey of dairy herds in the Moss Vale district of New South Wales. I. Disease wastage. Australian Veterinary Journal 44, 536-542.

McConnell, I., Munro, A. and Waldmann, H. (1981) The Immune System, 2nd edn. Blackwell Scientific Publications, Oxford. Pp. 252-255.

McFadden, G.K., Truitt, R.L. and Shechmeister, I.L. (1973) Ultrastructural examination of phytohemagglutinin stimulated lymphocytes infected with vesicular stomatitis virus. Arch. Gesamte Virusforsch 41(3), 229-237.

McFarland, H.F., Griffin, D.E. and Johnson, R.T. (1972) Specificity of the inflammatory response in viral encephalitis. 1. Adoptive immunization of immunosuppressed mice infected with Sindbis virus. The Journal of Experimental Medicine 136, 216-226.

Margolis, G. and Kilham, L. (1970) Parvovirus infection, vascular endothelium, and haemorrhagic encephalopathy. Laboratory Investigation 22, 478-88.

Mateva, V. and Feodorov, V. (1981) Ustanoviavane virusa na leukozata v limfotsiti na bolni goveda. Veterinarnomeditsinski nauki 18, 16-23.

Melik-Andreasian, G.G., Barinski, I.F., Guschin, B.V., Skorikova, A.S. and Labzo, S.S. (1981) Use of continuous human lymphoblastoid cell lines (T- and B-origin) to produce persistent tick-borne encephalitis virus and Venezuelan equine encephalomyelitis virus infections. Problems of Virology 2, 203-6.

Michaels, R.H. (1969) Immunologic aspects of congenital rubella. Pediatrics 43, 339-50.

Miles, J.A.R. (1952) An encephalitis isolated in South Australia. I. Some characteristics of the virus. Australian Journal of Experimental Biology and Medical Science 30, 341-351.

Miller, J.F.A.P. (1966) Immunity in the foetus and the newborn. British Medical Bulletin 22, 21-26.

Mims, C.A. (1968) Pathogenesis of viral infections of the fetus. Progress in Medical Virology 10, 194-237.

Mims, C.A. (1976) In The Pathogenesis of Infectious Disease. Academic Press, London.

Mims, C.A. and Tosolini, F.A. (1969) Pathogenesis of lesions in lymphoid tissue of mice infected with lymphocytic choriomeningitis (LCM) virus. British Journal of Experimental Pathology 50, 584-92.

Mitchell, R.M. (1959) Attempts to induce tolerance of renal homografts in sheep by intraembryonic injection of splenic cells. Transplantation Bulletin 6, 424-426.

Miyasaka, M. (1981) Immunological interaction between ewe and foetus. Thesis (Ph.D), Australian National University, Canberra.

Miyasaka, M. and McCullagh, P. (1981) Immunological responsiveness of maternal and foetal lymphocytes during normal pregnancy in the ewe. Journal of Reproductive Immunology 3, 15-27.

Miyasaka, M. and McCullagh, P. (1982) The response of the foetal lamb to maternal lymphocytes. Journal of Reproductive Immunology 4, 217-230.

Moore, N.W. and Rowson, L.E.A. (1958) Freemartins in sheep. Nature 182, 1754-1755.

Moore, N.W. and Rowson, L.E.A. (1961) Attempts to induce tissue tolerance in sheep. Research in Veterinary Science 2, 1-10.

Morgan, W.J.B. and Wrathall, A.E. (1977) In Infections and Pregnancy. Ed. C.R. Coid, Academic Press, London Pp.53-108.

Murphy, F.A., Taylor, W.P., Mims, C.A. and Marshall, I.D. (1973) Pathogenesis of Ross River virus infection in mice. II. Muscle, heart, and brown fat lesions. Journal of Infectious Diseases 127, 129-138.

Nairn, R.C. (1976) Fluorescent Protein Tracing, 4th edn. Churchill Livingstone, Edinburgh Pp. 162-277, 373.

Narita, M., Inui, S. and Hashiguchi, Y. (1979) The pathogenesis of congenital encephalopathies in sheep experimentally induced by Akabane virus. Journal of Comparative Pathology 89, 229-240.

Naryan, O. and Johnson, R.T. (1972) Effects of viral infection on nervous system development. I. Pathogenesis of bluetongue virus infection in mice. American Journal of Pathology 68, 1-14.

New, D.A.T. (1978) Whole embryo culture and the study of mammalian embryos during organogenesis. Biological Reviews 53, 81-122.

New, D.A.T. and Coppola, P.T. (1977) Development of a placental blood circulation in rat embryos in vitro. Journal of Embryology and Experimental Morphology 37, 227-235.

New, D.A.T., Mizzell, M. and Cockroft, D.L. (1977) Growth of opossum embryos in vitro during organogenesis. Journal of Embryology and Experimental Morphology 41, 111-123.

Niederhuber, J.E., Shermeta, D., Turcotte, J.G. and Gicas, P.W. (1971) Kidney transplantation in the fetal lamb. Technique and histopathology of rejection. Transplantation 12, 161-6.

Nossal, G.J.V. and Pike, B.L. (1980) Antibody receptor diversity and diversity of signals. Progress in Immunology IV 1, 136-151.

Nusbacher, J., Hirshhorn, K. and Cooper, L.Z. (1967) Chromosomal abnormalities in congenital Rubella. New England Journal of Medicine 276, 1409-1413.

Ohmann, H.B. (1982) Experimental foetal infection with bovine viral diarrhoea virus. II. Morphological reactions and distribution of viral antigen. Canadian Journal of Comparative Medicine 46, 363-369.

Ohmann, H.B. (1983) Pathogenesis of bovine viral diarrhoea-mucosal disease: Distribution and significance of BVDV antigen in diseased calves. Research in Veterinary Science 34, 5-10.

Ohmann, H.B. and Bloch, B. (1982a) Electron microscopic studies of bovine viral diarrhoea virus in tissues of diseased calves and in cell cultures. Archives of Virology 71(1), 57-74.

Ohmann, H.B., Jensen, M.H., Sorensen, K.J. and Dalsgaard, K. (1982b) Experimental infection with bovine viral diarrhoea virus. I. Virological and serological studies. Canadian Journal of Comparative Medicine 46, 357-362.

Olding, L.B., Jensen, F.C. and Oldstone, M.B. (1975) Pathogenesis of cytomegalo virus infection. I. Activation of virus from bone-marrow derived lymphocytes by in vitro allogenic reaction. Journal of Experimental Medicine 141(3), 561-572.

Oldstone, M.B. and Dixon, F.J. (1969) Pathogenesis of chronic disease associated with persistent lymphocytic chorio-meningitis viral infection. 1. Relationship of antibody production to disease in neonatally infected mice. Journal of Experimental Medicine 129, 483-505.

Olson, G.B., South, M.A. and Good, R.A. (1967) PHA un-responsiveness of lymphocytes from babies with congenital Rubella. Nature 214, 695-696.

Osburn, B.I. (1968) The relation of fetal age to the character of the lesions in fetal lambs infected with Brucella ovis. Pathologia Veterinaria (Basel) 5, 395-406.

Osburn, B.I. (1977) Sequential resistance to congenital infection. In Developmental Immunobiology, Eds. Soloman, J.B. and Horton, J.D. Elsevier/North Holland Biomedical Press, Amsterdam. Pp. 427-432.

Osburn, B.I. (1980) The ontogeny of the ruminant immune system and its significance in the understanding of maternal-fetal-neonatal relationships. In The Ruminant Immune System, Ed. Butler, J.E. Plenum Press, New York. Pp.91-103.

Osburn, B.I. and Hoskins, R.K. (1969) Antibody response to Brucella ovis in maternal and fetal sheep. Journal of Infectious Diseases 119, 267-272.

Osburn, B.I., Johnson, R.T., Silverstein, A.M., Prendergast, R.A., Jochim, M.M. and Levy, S.E. (1971a) Experimental viral-induced congenital encephalopathies. II. The pathogenesis of blue-tongue vaccine virus infection in fetal lambs. Laboratory Investigation 25, 206-210.

Osburn, B.I. and Kennedy, P.C. (1966) Pathologic and immunologic responses of the fetal lamb to Brucella ovis. Pathologia Veterinaria (Basel) 3, 110-136.

Osburn, B.I., Silverstein, A.M., Prendergast, R.A., Johnson, R.T. and Parshall, C.J. (1971b) Experimental viral-induced congenital encephalopathies. I. Pathology of hydranencephaly and porencephaly caused by blue tongue vaccine virus. Laboratory Investigation 25, 197-205.

Owen, 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 249, 361-363.

Owen, R.D. (1945) Immunogenetic consequences of vascular anastomoses between bovine twins. Science 102, 400-401.

Parsonson, I.M., Della-Porta, A.J., O'Halloran, M.L., Snowdon, W.A., Fahey, K.J. and Standfast, H.A. (1981a) Akabane virus infection in the pregnant ewe. I. Growth of virus in the foetus and the development of the foetal immune response. Veterinary Microbiology 6, 197-207.

Parsonson, I.M., Della-Porta, A.J., and Snowdon, W.A. (1977) Congenital abnormalities in newborn lambs after infection of pregnant sheep with Akabane virus. Infection and Immunity 15, 254-262.

Parsonson, I.M., Della-Porta, A.J., and Snowdon, W.A. (1981b) Akabane virus infection in the pregnant ewe. 2. Pathology of the foetus. Veterinary Microbiology 6, 209-224.

Parsonson, I.M., Della-Porta, A.J., Snowdon, W.A. and Murray, M.D. (1975) Congenital abnormalities in foetal lambs after inoculation of pregnant ewes with Akabane virus. Australian Veterinary Journal 51, 585-586.

Parsonson, I.M., Della-Porta, A.J., Snowdon, W.A. and O'Halloran, M.L. (1981c) Experimental infection of bulls with Akabane virus. Research in Veterinary Science 31, 157-160.

Parsonson, I.M., Della-Porta, A.J., Snowdon, W.A. and O'Halloran, M.L. (1981d) The consequences of infection of cattle with Akabane virus at the time of insemination. Journal of Comparative Pathology 91, 611-619.

Pearson, L.D., Doherty, P.C., Hapel, A. and Marshall, I.D. (1976) The responses of the popliteal lymph node of the sheep to Ross River and Kunjin viruses. Australian Journal of Experimental Biology and Medical Science 54, 371-379.

Peiris, J.S.M. and Porterfield, J.S. (1979) Antibody-mediated enhancement of Flavivirus replication in macrophage-like cell lines. Nature 282, 509-511.

Perryman, L.E., McGuire, T.C. and Torbeck, R.L. (1980) Ontogeny of lymphocyte function in the equine fetus. American Journal of Veterinary Research 41, 1197-1200.

Phillips, C.A., Melnick, J.L., Yow, M.D., Bayatoud, M. and Burkhardt, M. (1965) Persistence of virus in infants with congenital Rubella and in normal infants with a history of maternal Rubella. Journal of the American Medical Association 193, 1027-1029.

Phillips, R.A. and Melchers, P. (1976) Appearance of functional lymphocytes in fetal liver. Journal of Immunology 117, 1099-1103.

Plotkin, S.A., Boué, A. and Boué, J.G. (1965) The in vitro growth of Rubella virus in human embryo cells. American Journal of Epidemiology 81, 71-85.

Plotkin, S.A., Klaus, R.M. and Whitely, J.P. (1966) Hypogammaglobulinaemia in an infant with congenital Rubella syndrome: failure of 1-adamatanamine to stop virus excretion. Journal of Pediatrics 69, 1085-1091.

Plotkin, S.A. and Vaheri, A. (1967) Human fibroblasts infected with Rubella virus produce a growth inhibitor. Science 156, 659-661.

Poskitt, D.C., Frost, H., Cahill, R.N.P. and Trnka, Z. (1977) The appearance of non-specific antibody-forming cells in the efferent lymph draining antigen-stimulated single lymph nodes. Immunology 33, 81-89.

Rawls, W.E. (1968) Congenital rubella. The significance of virus persistence. Progress in Medical Virology 10, 238-285.

Rawls, W.E. (1974) Viral persistence in congenital rubella. Progress in Medical Virology 18, 273-88.

Reif, A.E. and Allen, J.M.V. (1964) The AKR thymic antigen and its distribution in leukaemias and nervous tissues. Journal of Experimental Medicine 120, 413-433.

Rice, C.E. and Silverstein, A.M. (1964) Haemolytic complement activity of sera of foetal and newborn lambs. Canadian Journal of Comparative Medicine and Veterinary Science 28, 34-37.

Robinson, J.H. (1983) The ontogeny of antigen-presenting cells in fetal thymus evaluated by MLR stimulation. The Journal of Immunology 130, 1592-1595.

Rosenberg, N., Baltimore, D. and Scher, C.D. (1975) In vitro transformation of lymphoid cells by Abelson murine leukemia virus. Proceedings of the National Academy of Science 72(5), 1932-1936.

Rosenberg, G.L., Farber, P.A. and Notkins, A.L. (1972) In vitro stimulation of sensitized lymphocytes by herpes simplex virus and vaccinia virus. Proceedings of the National Academy of Science U.S.A. 69, 756-760.

Sawyer, M., Moe, J.B. and Osburn, B.I. (1978) Ontogeny of immunity and leukocytes in the ovine foetus and elevation of immunoglobulins related to congenital infection. American Journal of Veterinary Research 39, 643-648.

Schechter, B., Handzel, Z.T., Altman, Y., Nir, E. and Levin, S. (1977) Cellular immunity in newborn infants and children - stimulation of lymphocyte protein synthesis as a measure of immune competence. Clinical Experimental Immunology 27, 478-484.

Sellers, M.I. (1969) Studies on the entry of viruses into the central nervous system of mice via the circulation. Differential effects of vasoactive amines and CO_2 on virus infectivity. Journal of Experimental Medicine 129, 719-746.

Shepherd, N.C., Gee, C.D., Jessep, T., Timmins, G., Carroll, S.N. and Bonner, R.B. (1978) Congenital bovine epizootic arthrogryposis and hydranencephaly. Australian Veterinary Journal 54, 171-177.

Sherwin, W.K. and Rowlands, D.T. (1975) Determinants of the hierarchy of humoral immune responsiveness during ontogeny. Journal of Immunology 115, 1549-1554.

Siewers, C.M.F., John, C.E. and Medearis, D.N. (1970) Sensitivity of human cell strains to interferon. Proceedings of the Society for Experimental Biology and Medicine 133, 1178-1183.

Silverstein, A.M. (1972)

In Ontogeny of Acquired Immunity. Ciba Foundation Symposium, Elsevier, Amsterdam Pp. 17-25.

Silverstein, A.M. and Prendergast, R.A. (1970) Lymphogenesis, immunogenesis, and the generation of immunologic diversity. In Developmental Aspects of Antibody Formation and Structure. Eds. Sterzl, J. and Riha, I. Academia, Prague Pp. 69-77.

Silverstein, A.M., Prendergast, R.A. and Kraner, K.L. (1964) Foetal response to antigenic stimulus. IV. Rejection of skin homografts by the fetal lamb. Journal of Experimental Medicine 119, 955-964.

Silverstein, A.M., Thorbecke, G.J., Kraner, K.L. and Lukes, R.J. (1963a) Foetal response to antigenic stimulus. III. Gammaglobulin production in normal and stimulated fetal lambs. Journal of Immunology 91, 384-395.

Silverstein, A.M., Uhr, J.W., Kraner, K.L. and Lukes, R.J. (1963b) Fetal response to antigenic stimulus. II. Antibody production by the fetal lamb. Journal of Experimental Medicine 117, 799-812.

Simpson-Morgan, M.W., Trevella, W., Hugh, A.R., McClure, S.J. and Morris, B. The long-term collection of lymph from single lymph nodes of foetal lambs in utero. (In preparation).

Singer, D.B., Rudolph, A.J., Rosenberg, H.S., Rawls, W.E. and Boniuk, M. (1967) Pathology of the congenital Rubella syndrome. Journal of Pediatrics 71, 665-675.

Smith, H., Williams, A.E., Pearce, J.H., Keppie, J., Harris-Smith, P.W., Fitz-George, R.B. and Witt, K. (1962). Foetal erythritol: a cause of the localization of Brucella abortus in bovine contagious abortion. Nature 193, 47-49.

Solomon, J.B. (1971) Foetal and neonatal immunology. North-Holland, Amsterdam. Pp. 155-189.

Solomon, J.B., Barrow, A. and Jappy, A. (1977) Immunological milestones in ontogeny of the guinea-pig. In Developmental Immunobiology. Ed. Solomon, J.B. and Horton, J.D. Elsevier/North Holland, Amsterdam. Pp. 441-448.

Spradbrow, P.B. and Clark, L. (1966) Experimental infection of calves with a group B arbovirus (Kunjin virus). Australian Veterinary Journal 42, 65-66.

Spurr, A.R. (1969) A low viscosity epoxy resin embedding medium for electron microscopy. Journal of Ultrastructure Research 26, 31-43.

Steele, C.E. (1972) Improved development of rat 'egg-cylinders' in vitro as a result of fusion of the heart primordia. Nature New Biology 237, 150-151.

Steele, C.E. and New, D.A.T. (1974) Serum variants causing the formation of double hearts and other abnormalities in explanted rat embryos. Journal of Embryology and Experimental Morphology 31, 707-719.

Steven, D.H. (1975) Anatomy of the placental barrier. In Comparative Placentation: essays in structure and function. Ed. Steven, D.H. Academic Press, London. Pp. 25-56.

Steven, D.H. and Morriss, G. (1975) Development of the foetal membranes. In Comparative Placentation. Ed. Steven, D.H. Academic Press, London. P. 59.

St. George, T.D., Standfast, H.A. and Cybinski, D.H. (1978) Isolations of Akabane virus from sentinel cattle and Culicoides brevitarsis. Australian Veterinary Journal 54, 558-561.

Stein, G.H. and Yanishevsky, R. (1979) Autoradiography. In Methods in Enzymology, Vol. LVIII. Ed. Jakoby, W.B. and Pastan, I.M. Academic Press, Pp. 279-292.

Stoerker, J., Yajima, Y. and Glaser, R. (1982) The interaction of non-transforming Epstein-Barr virus (EBV) with cell-associated EBV DNA in superinfected lymphoblastoid cell lines. Journal of Cancer 30 , 385-392.

Stott, J.L. and Osburn, B.I. (1980) Humoral and cellular immune response of sheep and cattle to acute bluetongue virus infection. Advances in experimental medicine and biology 137, 828.

Summers, B.A., Greisen, H.A. and Appel, M.J. (1978) Possible initiation of viral encephalomyelitis in dogs by migrating lymphocytes infected with distemper virus. Lancet 2(8082), 187-189.

Symons, D.B.A. and Binns, R.M. (1975) Immunoglobulin-bearing lymphocytes: their demonstration in adult sheep and ontogeny in the sheep fetus. International Archives of Allergy and Applied Immunology 49, 658-669.

Tajima, M. and Ushijima, T. (1971) The pathogenesis of rinderpest in the lymph nodes of cattle. American Journal of Pathology 62, 221-228.

Taussig, M.J. (1979) In Processes in Pathology. Blackwell, Oxford, P. 75.

Terpstra, C. (1981) Border disease: virus persistence, antibody response and transmission studies. Research in Veterinary Science 30, 185-191.

Tervit, H.R., Whittingham, D.G. and Rowson, L.E.A.(1972) Successful culture in vitro of sheep and cattle ova. Journal of Reproduction and Fertility 30, 493-497.

Teute, H., Braun, R., Kirchner, H. and Munk, K. (1983) Replication of Herpes simplex virus in human T lymphocytes. Intervirology 20/1, 32-41.

Toivanen, P., Asantila, T., Granberg, C., Leino, A. and Hirvonen, T. (1978) Development of T cell repertoire in the human and the sheep foetus. Immunological Reviews 42, 185-201.

Tondury, G. and Smith, D.W. (1966) Fetal Rubella pathology. Journal of Pediatrics 68, 867-879.

Traub, E. (1939) Epidemiology of lymphocytic choriomeningitis in a mouse stock observed for four years. Journal of Experimental Medicine 69, 801-817.

Trnka, Z. and Cahill, R.N.P. (1980) Aspects of the immune response in single lymph nodes. Monographs in Allergy 16, 245-259.

Umiel, T., Globerson, A. and Auerbach, R. (1968) Role of the thymus in the development of immunocompetence of embryonic liver cells *in vitro*. *Proceedings of the Society for Experimental Biology and Medicine* **129**, 598-600.

Upcott, D.H., Hebert, C.N. and Robins, M.(1972) Erythrocyte and leukocyte parameters in fetal lambs. *Research in Veterinary Science* **13**, 507-510.

Urbianak, S.J., White, A.G., Barclay, G.R., Wood, S.M. and Kay, A.B. (1978) Tests of immune function. In *Handbook of Experimental Immunology*, 3rd edn. Ed. Weir, D.M. Blackwell, Oxford. Pp. 47.1-47.31.

Wainberg, M.A., Vydelingum, S. and Margolese, R.G. (1983) Viral inhibition of lymphocyte mitogenesis: interference with the synthesis of functionally active T cell growth factor(TCGF) activity and reversal of inhibition by the addition of same. *Journal of Immunology* **130**, 2372-2378.

Waithe, W.I. and Hirschhorn, K. (1978) Lymphocyte response to activators. In *Handbook of Experimental Immunology*, 3rd edn. Ed. Weir, D.M. Blackwell, Oxford Pp. 26.1-26.9.

Wales, R.G. and Murdoch, R.N. (1973) Changes in the composition of sheep fetal fluids during early pregnancy. *Journal of Reproduction and Fertility* **33**, 197-205.

Wanner, R.A. (1975) A study of the effects of hyperthermia and certain other agents on the developing brain of the guinea-pig. Ph.D. Thesis, The University of Sydney.

Wardley, R.C. (1982) Effect of African Swine Fever on lymphocyte mitogenesis. *Immunology* **46**, 215-220.

Westbury, H.A., Napthine, D.V. and Straube, E. (1979) Border disease: persistent infection with the virus. Veterinary Record 104, 406-409.

White, L.R., Leikin, S., Villavicencio, O., Abernathy, W., Avery, G. and Sever, J.L. (1968) Immune competence in congenital rubells: lymphocyte transformation, delayed hypersensitivity, and response to vaccination. Journal of Pediatrics 73, 229-34.

Whittem, J.H. (1957) Congenital abnormalities in calves: arthrogryposis and hydranencephaly. Journal of Pathology and Bacteriology LXXIII, 375-387.

Wimsatt, W.A. (1950) New histological observations on the placenta of the sheep. American Journal of Anatomy 87, 391-457.

Young, S. and Cordy, D.R. (1964) An ovine fetal encephalopathy caused by bluetongue vaccine virus. Journal of Neuropathology and Experimental Neurology 23, 635-659.

Young, G.A., Kitchell, R.L., Luedke, A.J. and Sautter, J.H. (1955) The effect of viral and other infections of the dam on fetal development in swine. I. Modified live hog cholera viruses - immunological, virological, and gross pathological studies. Journal of the American Veterinary Medical Association 126, 165.

Zakarian, B., Barlow, R.M. and Rennie, J.C. (1975) Periarteritis in experimental Border disease of sheep. 1. The occurrence and distribution of the lesion. Journal of Comparative Pathology 85, 453-460.

Zakarian, B., Barlow, R.M., Rennie, J.C. and Head, K.W. (1976) Periarteritis in experimental Border disease of sheep. II. Morphology and histochemistry of the lesion. Journal of Comparative Pathology 86, 477-487.

Zanders, E.D., Smith, C.M. and Callard, R.E. (1981) A micro-method for the induction and assay of specific in vitro antibody responses by human lymphocytes. Journal of Immunological Methods 47, 333-338.

Zinkernagel, R.M. and Doherty, P.C. (1974) Immunological surveillance against altered self components by sensitized T lymphocytes in lymphocytic choriomeningitis. Nature 251, 547-548.