IMMUNOLOGICAL INTERACTION

BETWEEN EWE AND FOETUS.

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MASAYUKI MIYASAKA

Department of Immunology,

John Curtin School of Medical Research,

Australian National University,

Canberra, Australia.

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STATEMENT.

Dr. Peter McCullagh assisted me in undertaking foetal operations. With this exception, the experiments described in this thesis were done entirely by myself.

Masayuki MIYASAKA

To my parents

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SUMMARY

In this project, the immunological competence of a series of ewes was monitored throughout pregnancy. Although the reactivity of maternal lymphocytes against paternal and third-party PBL and Con A was not observed to vary significantly, there was a marked increase in the responsiveness of lymphocytes from some ewes towards foetal thoracic duct, paternal and third-party efferent lymphatic lymphocytes and LPS during pregnancy. The stimulatory capacity of maternal PBL for paternal or foetal cells remained quite constant throughout pregnancy. The reactivity of lymphocytes returning from the gravid uterus was indistinguishable from that of lymphocytes from other regions of the maternal circulation. Whilst sera collected throughout pregnancy from a group of ewes did not interfere with the reactivity of maternal lymphocytes *in vitro*, some plasma specimens collected in the later stages of pregnancy did reduce these responses. Cytotoxic activity could not be demonstrated in any of the plasma specimens collected.

Ten pregnant ewes manifested significantly increased lymphocyte output followed by an increase in lymphoblast output from the popliteal lymph node in response to challenge with foetal or paternal lymphocytes between 93 and 127 days. Cytotoxic antibody appeared in the lymph in every instance in which the popliteal cannula remained patent for a week. Both anti-paternal and anti-foetal MLC reactivity of cells efferent from the stimulated node, in addition to their responsiveness to Con A, were severely depressed as the cell output from the node increased. Cytotoxic cells could neither be detected in the efferent lymph from stimulated maternal nodes nor generated from normal cells *in vitro*.

Four out of 22 lambs which had been exposed to allogeneic or maternal lymphocytes *in utero* (49 - 73 days gestation) survived

to full term. These lambs showed normal MLC responsiveness towards the cell donors and rejected skin grafts from the priming animals. However, serum samples from 3 lambs inhibited the MLC response of autologous cells to cells from both the priming and third-party animals and 2 lambs responded abnormally in the NLT test.

Foetal lymphocytes (collected from 110 to 134 days old foetuses) behaved indistinguishably from adult cells in vitro and foetal lamb plasma did not affect the responsiveness of autologous lymphocytes in vitro.

Four out of 9 foetuses which received large numbers of unstimulated maternal cells in utero (110 to 117 days) survived to term and showed no pathological signs. In contrast, all foetuses that had received large numbers of maternal lymphocytes specifically sensitized against foetal or paternal cells died very rapidly. Cytotoxic antibody was not detected in any of the challenged foetuses, but thoracic duct cells collected from some showed markedly depressed anti-maternal MLC reactivity, a change which appeared to be specific in some instances.

The current study has excluded the occurrence of any specific modification of the reactivity of those maternal lymphocytes passing through the circulation of the gravid uterus and placental tissues and consequently suggests that an effective placental separation of the two circulating cell populations is a major factor in the maintenance of foetal-maternal homeostasis. Additionally, the extent of foetal competence in responding to maternal cells suggests the existence of an additional mechanism to protect the lamb against transplacental passage of these cells.

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GLOSSARY OF TERMS

AFP	Alpha-fetoprotein
ALS	Anti-lymphocyte serum
Allo BM	Allogeneic bone marrow
Allo eff	Allogeneic efferent lymphatic cells
B cell	Bone marrow derived cell
Con A	Concanavalin A
cpm	count per minute
D-MEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulphoxide
DNP-Ficoll	ε-Dinitrophenyl-lysine-Ficoll
EDTA	Ethylenediaminetetraacetic acid
EPF	Early pregnancy factor
F	Foetal
FACS	Fluorescence activated cell sorter
FCS	Foetal calf serum
FLP	Foetal lamb serum
GVHD	Graft-versus-host disease
GVHR	Graft-versus-host reaction
Hanks' BSS	Hanks' balanced salt solution
HCG	Human chorionic gonadotropin
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
Ia antigen	I region associated antigen
LPS	Lipopolysaccharide
Mat LN	Maternal lymph node
MLC	Mixed lymphocyte culture
MLR	Mixed lymphocyte reaction
n.d.	Not determined

N.S.	No sample
NLT	Normal lymphocyte transfer
P	Paternal
Pat eff	Paternal efferent lymphatic cells
Pat PBL	Paternal peripheral blood lymphocytes
PBL	Peripheral blood lymphocytes
PHA	Phytohaemagglutinin
pp	Post partum
PPO	2,5-Diphenyloxazole
PWM	Pokeweed mitogen
S.D.	Standard deviation
S.E.	Standard error
SRBC	Sheep red blood cells
T cell	Thymus derived cell
TDL	Thoracic duct lymphocytes
TNCB	Trinitrochlorobenzene

INTRODUCTION

A paradoxical immunological aspect of pregnancy, the failure of the mother to mount an immune response against its antigenically foreign foetus, has been the subject of much speculation (Kirby 1968, Beer and Billingham 1976, Loke 1978). Medawar (1953), in reviewing the subject, suggested 3 possible mechanisms to account for acceptance of the foetus by its mother.

(1) Anatomical separation of the foetus from its mother may be sufficient to prevent sensitization of either against the other.

(2) The foetal tissues may be antigenically immature.

(3) The immune system of the pregnant animal may be inert.

Many subsequent experimental and clinical studies have been directed to testing these suggestions.

This chapter reviews the information currently available on foetal antigenicity, foetal reactivity and its ontogeny, maternal reactivity, immunological interactions between mother and foetus and their possible importance. It also attempts to gauge the significance of all of these factors for successful completion of pregnancy.

A. ANTIGENICITY OF SEMEN AND FOETAL TISSUES

1) Antigenicity of Semen

Seminal antigens can be classified into two groups; (1) antigens found on spermatozoa and in the testis and epididymis, (2) those of the adnexal glands and their secretions. Antigens detectable on spermatozoa may be either true membrane antigens or antigens absorbed from seminal plasma. All of these antigens can also be categorized into two separate groups;

(1) tissue-specific or auto-antigens against which both the male himself and the female recipient can respond,

(2) alloantigens against which a female recipient can react.

The existence of both allo- and auto-antigens of semen has been reported from early in the century in independent studies by Landsteiner, Metchnikoff, Metalnikoff and Adler (reviewed by Shulman 1975). The autoantigenicity of semen was confirmed by the later studies of Freund (Freund, Lipton and Thompson 1953, Freund, Thompson and Lipton 1955) and of Voisin's group (Toullet, Voisin and Nemirovsky 1970). Seminal alloantigenicity was originally demonstrated by interspecies immunization but, more recently, a variety of methods have been used to demonstrate alloantigens on spermatozoa or in seminal plasma. The presence of blood group antigens on sperm was suggested by Landsteiner and Levine (1926) by simple absorption methods and later confirmed by Gullbring (1957) using a mixed agglutination technique and by Shahani and Southam (1962) by means of immunofluorescence. Although it is still uncertain whether these are structural or absorbed antigens (Parish, Carron-Brown and Richards, 1967, Isojima and Tsuzuku, 1968), their significance for pre-zygotic selection is questionable because spermatozoa were not immobilised, agglutinated or lysed by isoagglutinins (Isojima and Tsuzuku 1968).

The presence of histocompatibility antigens has been demonstrated (Fellous and Dausset 1970) and cytotoxicity studies suggest the possibility that haploid expression of HL-A antigens may occur on human spermatozoa (Fellous and Dausset 1970, Halim, Abbasi and Festenstein 1974) but this suggestion awaits further confirmation. Soluble HL-A substances have been demonstrated in seminal plasma by a cytotoxicity inhibition assay (Singal, Berry and Naipaul, 1971).

The immunogenicity of seminal antigens in the environment of the female reproductive tract has been studied because of its potential use in contraception. This idea was originally highlighted by Charles Darwin in 1871 (cited by Kirby 1968). Darwin held the view that "the diminution of fertility may be explained in some cases by the profligacy of the

women.". Experimental immunization of females with sperm or testis homogenate has been shown to reduce fertility in various species (reviewed by Johnson, Hekman and Rümke 1975) and studies on inbred mice show that an immune reaction of the female against tissue- or male-specific antigens can result in a reduction in fertility (Bell and McLaren 1970). However, this effect was dependent upon prolonged immunization or the use of an adjuvant. Beer and Billingham (1976) provided strong evidence for the alloantigenicity of spermatozoa by successfully inducing transplantation immunity in female rats, mice and hamsters by injecting washed allogeneic spermatozoa into their uterus. Although they claimed that contaminating leucocytes and epithelial cells in the inocula were not responsible for this phenomenon as the number of these cells was small, this point requires further experimental verification.

The occurrence of antibodies to spermatozoa has been associated with human infertility. Although correlation between the titres of antisperm antibodies and infertility is still unclear (Beer and Billingham 1976), it appears that these antibodies may play a role in some cases of otherwise unexplained infertility. In general, coitally-induced sensitization of the female appears to be rare and the observation that spermatozoa, if dispensed in seminal plasma and inoculated into the uterus of mice, failed to incite a generalized state of transplantation immunity in female mice (Beer and Billingham 1976) may be relevant.

Unequivocal evidence of infertility resulting from an immune response to allo- or auto-antigens of accessory gland tissues and secretions is lacking (Johnson, Hekman and Rümke 1975).

Other than causing immunity, it has been suggested that seminal antigens are able to induce a state of tolerance in the female under certain experimental conditions. Prehn (1960) reported prolonged survival of syngeneic male skin grafts on C57BL females which had been repeatedly

exposed to non-sperm cells and seminal plasma by mating with sterile males. Lengerová and Vojtisková (1963) obtained similar results in C57BL female mice, the fallopian tubes of which had been ligated before repeated mating with normal C57BL males. However, the relevance of this phenomenon for fertility is unknown.

2) Antigenicity of Foetal Tissues

Little (1924) claimed that the foetus is deficient in antigenicity until relatively late in its development and this notion was included in Medawar's attempts to account for the successful coexistence of mother and foetus (1953). However, later studies have not confirmed this notion. Palm, Hyner and Brinster (1971) demonstrated the presence of minor histocompatibility antigens on the two-cell mouse embryo with an immunofluorescence technique. Searle et al. (1976), employing an immunoperoxidase technique, reported that there were detectable amounts of H-2 antigens on mouse blastocysts. Krco and Goldberg (1977) demonstrated H-2 antigens on 8-cell mouse embryos using complement dependent cytotoxic assays. Epstein, Smith and Travis (1980) demonstrated H-Y antigen on the 8-cell mouse embryo by karyotyping viable embryos following teatment of the embryos with anti-H-Y serum and complement.

There is ample evidence for antigenicity of human foetal tissues. Skin grafts from foetuses of from 17 to 33 weeks gestation were all rejected in a normal fashion when transplanted to unrelated recipients (Goldstein and Baxter 1958). Lymphocytes obtained from foetuses of from 10 to 26 weeks gestation can be typed for HL-A antigens (Ceppellini et al. 1971). Lymphocytes collected from 16 to 24 weeks foetuses can respond to and stimulate adult lymphocytes in a one-way MLC (Pegrum 1971). Anti-HL-A antibodies can usually be demonstrated by about the 24th week of pregnancy in primiparous women (Ahrons 1970a).

Obviously, the foetus expresses antigenicity to which the mother can

respond immunologically and Little's hypothesis (1924) is invalid. However, the possibility remains that the extra-embryonic tissues which surround the foetus could be non-immunogenic and so act as a 'barrier' between mother and foetus.

3) Antigenicity of Trophoblast

The trophoblast of different species establishes varying degrees of physical contact with the uterine tissue and circulating maternal blood cells depending upon the type of placenta (to be discussed later). In the human, trophoblast cells are directly in contact with maternal blood and can become separated and enter the maternal circulation (up to 100,000 cells/day) (Edwards and Coombs 1975).

The immunogenicity of the human trophoblast in vivo is controversial. There are reports that anti-trophoblast antibody has been detected in postpartum (Hulka et al. 1963) and pregnant women (Burstein and Blumenthal 1969) but Green and Urback (1968) failed to detect any antibody activity against trophoblast in maternal postpartum sera. Youtanannkorn and Matangkasombut (1972, 1973) using the macrophage migration inhibition test reported that leucocytes from postpartum women could inhibit the migration of guinea pig peritoneal macrophages in the presence of 'placental' antigens. However, the antigen used was obtained by enzyme digestion of placental membranes and contamination with antigens derived from non-trophoblastic tissues cannot be excluded.

Currie and Bagshawe (1967) obtained trophoblastic cells by trypsinizing human chorionic villi and showed that maternal lymphocytes as well as lymphocytes from unrelated donors were cytotoxic for these trophoblastic cells grown in culture. The phenomenon only occurred with allogeneic cells. Similar results have been obtained by others (Douthwaite and Urbach 1971, Taylor and Hancock 1975). Loke, Joysey and Borland (1971) reported that trophoblastic cells, similarly grown in culture, were killed in the presence

of anti-HL-A serum and complement. However, these results cannot be accepted as cogent evidence for the presence of alloantigens on the trophoblast because of uncertainty about the identity of cells prepared in these ways. Seigler and Metzger (1970) found that trophoblast syncytium failed to absorb alloantisera. Faulk, Sanderson and Temple (1977) could not demonstrate HL-A, β_2 microglobulin or Ia antigens on trophoblast by means of immunofluorescence staining, a result that was also obtained by Sundgvist, Bergström and Håkansson (1977).

Currie, Van Doorninck and Bagshawe (1968) reported that exposure to neuraminidase rendered mouse ectoplacental cones as antigenic as spleen cells, but this observation has not been confirmed by later studies (Simmons et al. 1971, Searle et al. 1975). Thus, while the possibility remains that the trophoblast carries alloantigen in a masked form, it is more likely that trophoblast is non-immunogenic and, as such, could form a barrier between mother and foetus. Other possible mechanisms for trophoblast non-antigenicity have been reveiwed by Simmons (1972) and by Loke (1978).

If preimplantation eggs or blastocysts do express antigenicity, it may be necessary to postulate the existence of some mechanism for their survival before formation of the trophoblastic barrier. James (1969) showed that mouse blastocysts were resistant to immune sera and complement only if the zona pellucida was intact. Trounson, Pugh and Moore (1976) showed that an intact zona pellucida reduced the susceptibility of sheep ova to immune lysis by antisera raised against ovary, oocytes and sperm and that the zona was impermeable to antibodies tagged with fluorescein. In contrast, Sellens and Jenkinson (1975) reported that the zona of mouse ova was readily permeable to antibodies and provided little protection against immune lysis. The extent to which this difference reflects species differences is not clear.

B. FOETAL AND NEONATAL REACTIVITY

The subject of immunological competence of the foetus has been extensively studied using a variety of species of animals. The extent of variations between species necessitates separate review of each species and an attempt will be made to review studies of 6 representative species (rat and mouse, rabbit, guinea pig, sheep, human).

1) Rat and Mouse (gestation period 21 days)

The development of transplantation immunity in these species has been investigated extensively. The period after birth during which tolerance (as indicated by subsequent skin graft survival) can be induced is quite variable (often up to a week in the rat) depending on the strain of the donor and recipient, their genetic proximity, the type and number of cells transferred, and the route of transfer (Billingham, Brent and Medawar 1953, 1956, Billingham and Brent 1956, 1957, Medawar and Woodruff 1958, Billingham and Silvers 1962).

A few hypotheses have been put forward to account for inability of newborns of these species to respond to a variety of antigens. Nossal (1959) reported that spleen cells from normal adult rats and mice responded poorly to challenge after transfer to intact or irradiated neonates, whereas cells from immune donors could respond provided they had been incubated with antigen before transfer. This inability to sustain primary immune responses was tentatively attributed to functional immaturity of the neonate's macrophages. Argyris (1968) had demonstrated that the transfer of syngeneic peritoneal cells from thioglycollate-stimulated adult mice to 1 day-old C3H mice can augment the immunological reactivity of the recipients to sheep red blood cells (SRBC) when this is tested 3 days later, suggesting that neonatal macrophages are immature. Nakano, Hosokawa and Muramatsu (1978) and Nakano, Aotsuka and Muramatsu (1978) assessed the function of macrophages from donors of

different ages by their capacity to restore the *in vitro* anti-SRBC response of macrophage-depleted cell populations and found a good correlation between the development of responsiveness of intact C3H mice to SRBC and the development of ability of their macrophages to restore the *in vitro* response. Blaese and Lawrence (1977) made an observation similar to that of Argyris (1968) in Lewis rats but also observed that transfer of adult peritoneal exudate cells into newborn rats failed to augment their anti-pneumococcal polysaccharide responsiveness indicating that impaired immune reactivity in the neonatal period cannot be explained by immature macrophage function alone.

McCullagh (1975a, 1975b) proposed that there was a dominant state responsible for suppressing immune responses in neonatal animals and that the suppression was mediated by thymus-derived cells. He observed that, although newborn rats were unable to sustain an adoptive immune response against SRBC following the transfer of normal thoracic duct lymphocytes, irradiation of the neonates at levels as low as 350 rads was effective in overcoming the resistance and that thymectomy performed on the day of birth gave an effect similar to irradiation. Although the precise mechanism of the suppression is not known, this is clear evidence for active interference with some aspect of the immune response in the neonate.

Reports on suppressor cells in foetal or newborn animals have appeared sporadically since. The ability of paternal strain lymphocytes to elicit local GVHR in F_1 hybrid strain animals is significantly reduced by the addition of foetal spleen cells (but not thymocytes) to the inocula of paternal cells and there is no requirement for histocompatibility between the lymphocytes and spleen cells (Skowron-Cendrzak and Ptak 1976). Foetal or neonatal spleen cells are able to suppress the passive transfer by adult cells of contact sensitivity to picryl chloride but it is not known whether histocompatibility between adult and neonatal cells is required for this effect (Ptak and Skowron-Cendrzak 1977). Umiel, Globerson and Trainin (1977)

reported suppressive activity of neonatal liver cells both in vitro and in vivo. In their in vitro system, neonatal liver cells were found to exert a nonspecific suppressive effect in the MLC. In the in vivo system, the suppressive activity of neonatal liver cells was assessed by their ability to interfere with the GVH activity of C57BL/6 spleen cells which had been introduced into sublethally irradiated (C3H x C57BL/6)F_1 hybrid mice. Immunological specificity was demonstrable as GVHR was prevented only if the GVH-inducing spleen cells and the neonatal liver cells were syngeneic. Furthermore, interference with the GVH was observed only if the recipient possessed a thymus. Consequently they suggested that the neonatal liver contained precursors of suppressor cells which could differentiate only in the presence of the thymus. However, the probability was that neonatal liver cells would also act as a source of haematopoietic cells which might be more important than any suppressor cells in the protection of F_1 hybrid animals from a fatal GVHR. Mosier and Johnson (1975) also reported neonatal suppressor cells employing an approach in which the effect of neonatal (2 week-old) T cells on in vitro antibody synthesis by adult spleen cells was assessed. Their conclusion was based on two observations, namely that adult T cells failed to cooperate with neonatal B cells to form IgM in response to SRBC in vitro unless neonatal T cells were first removed and that neonatal splenic T cells interfered with the ability of adult B cells to respond to DNP-Ficoll. However, there are some obstacles to these interpretations as 2 week-old mice from which the 'neonatal suppressor cells' were collected responded well to DNP-Ficoll. Furthermore, neonatal spleen cells, although unable to synthesize IgM in vitro, respond well to DNP-Ficoll when transferred to irradiated recipients (Chiller 1979).

In summary, although evidence for the operation of a suppressive mechanism in some situations in the neonate is unequivocal, this is clearly not the only factor in the inability of the neonate to respond to antigens.

However, the extraordinarily early development of activity against those maternal lymphocytes with anti-foetal potential in F_1 hybrid animals compared with the age of appearance of other alloreactivities (McCullagh 1977) may reflect a biological relevance of this response in evolution.

2) Rabbit (gestation period 32 days)

Porter (1960) injected allogeneic spleen cells into the foetuses and observed that rejection of skin grafts from the cell donor (applied at 8 weeks after birth) was prolonged if the foetus had been inoculated at the 22nd day of gestation, but accelerated if challenge had been given later than the 24th day of gestation. He concluded that the 'adaptive period' of the foetal rabbit ends by the 24th day of gestation. Najarian and Dixon (1962) have reported that neonatal rabbits were capable of launching an allograft reaction more rapidly than adults. Dixon and Weigle (1957) showed that adult lymph node cells which were capable of forming antibody if transferred into adult or irradiated adults failed to produce antibody if injected into neonatal rabbits. Similarly, Warwick, Archer and Good (1960) failed to transfer delayed hypersensitivity to streptococci into 7 day-old rabbits. These results could be explained by immaturity of macrophage function or lack of collaborator cells in the neonate (the presence of collaborator cells in animals which were themselves unresponsive as a result of irradiation was shown by Ellis, Gowans and Howard (1969)), or an active suppression mechanism as proposed by McCullagh (1975a, 1975b), or by a superior alloreactivity of the neonates over adult animals as reported by Najarian and Dixon (1962) (as the rabbits used were not inbred, the neonates could have rejected the transferred cells).

3) Guinea pig (gestation period 65-70 days)

Egdahl established that, if foetal guinea pigs were grafted from 1 to 3

days before birth, they could reject skin allografts at about the same rate as adults (cited by Solomon 1971). Mesenteric lymph node cells collected from newborns and foetuses within a week of term expressed normal activity in the normal lymphocyte transfer (NLT) test (Brent and Medawar 1962). However, early studies suggested that young guinea pigs were unable to respond as readily as adults in delayed hypersensitivity reactions (Freund 1927, 1929). Salvin, Gregg and Smith (1962) confirmed Freund's early work but demonstrated the presence of sensitized cells in newborns by means of the passive transfer of delayed hypersensitivity from sensitized newborns to normal adults. This report and an earlier report by Waksman and Matoltsy (1958) of the failure of attempts at passive transfer of delayed hypersensitivity from adults to newborn guinea pigs suggest either the inability of neonatal skin to sustain allergic reactions or the presence of situations similar to those discussed in the preceding section.

There appears to be a variation in the age of attaining immunological maturity, as indicated by humoral antibody production, depending on the antigen employed (Solomon 1971).

4) <u>Sheep (gestation period 148-150 days)</u>

Schinckel and Ferguson (1953) were the first to demonstrate that foetal sheep possess immunological reactivity. Foetal lambs were observed to reject skin allografts placed between 80 and 117 days of age. The immune response of the foetal lamb has been extensively studied subsequently. The advantages of using sheep are

(1) surgical intervention need not prejudice the course of pregnancy,

(2) a long gestational period and a foetus sufficiently large for immunological reactions to be studied and

(3) the foetus develops in an antigen-free environment in the absence of transmitted maternal antibodies.

Silverstein, Prendergast and Kraner (1964) confirmed Schinckel and Ferguson's data (1953) and showed that skin allografts were invariably rejected with normal speed when applied to foetuses after day 77 of gestation. The process of rejection was not accompanied by the appearance of plasma cells in the draining lymphoid tissues or by serum immunoglobulin production. If grafts were applied about the 65th day of gestation, they were accepted and retained at least for up to 21 days, without any indication of active rejection processes. This suggested that transplantation immunity developed between 65 and 77 days. Niederhuber et al. (1971) reported the rejection of kidney allografts by foetal lambs of from 70 to 130 days gestation. Attempts to induce homograft tolerance in foetal lambs have been made without success (Mitchell 1959, Moore and Rowson 1961). Mitchell injected allogeneic spleen cells into 90 day embryos and transplanted kidneys from the spleen cell donors after birth. Two lambs which were inoculated _in utero_ thrived normally and rejected kidneys from the spleen cell donors after birth. Moore and Rowson (1961) used a total of 115 foetuses to test whether tolerance could be induced _in utero_. A variety of sources of lymphocytes such as adult lymph node cells, spleen cells, blood cells and foetal blood cells were injected into foetal lambs of from 50 to 110 days gestation. As in the experiments of Mitchell (1959), many foetuses were resorbed. Less than 40% of lambs that were between 50 and 60 days gestation at the time of injection, were born, whereas virtually all foetal lambs which had attained 80 or more days at the time of injection were born alive. One to 2 months later, these lambs were tested for tolerance by transfer of skin allografts from their respective donors. However, all were rejected within 10 days, suggesting that tolerance had not been induced. These results can be interpreted in at least 2 ways. Firstly the number of injected cells may not have been sufficient to induce tolerance, secondly, transplantation immunity may have developed before 50 days so that the inoculated cells were rejected by the foetus. A report of

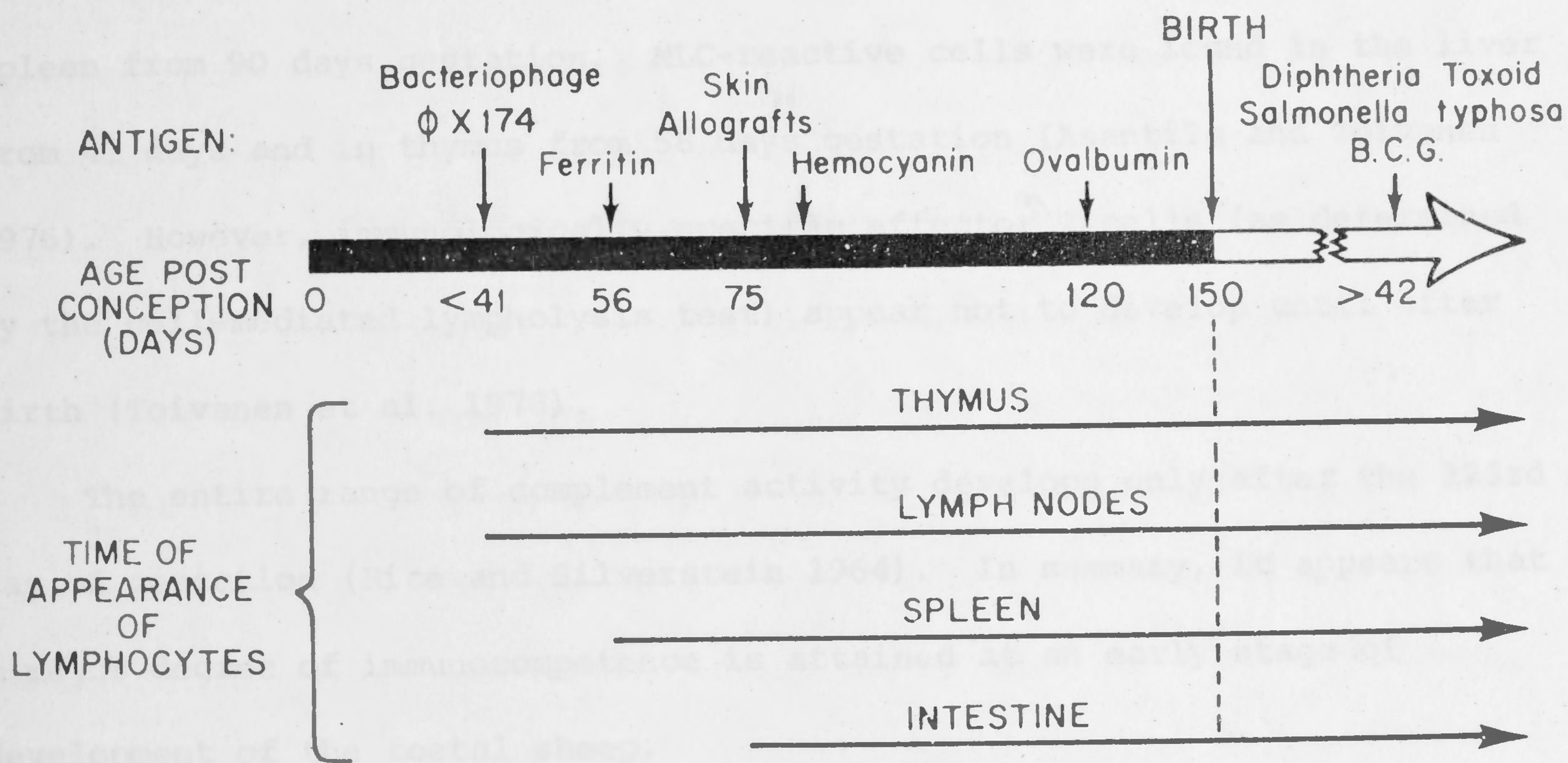
tolerance occurring in dizygotic sheep twins (Moore and Rowson 1958) suggests that tolerance can be induced naturally in rare occasions.

Silverstein has studied antibody synthesis by the foetal lamb (Silverstein, Uhr and Kraner 1963, Silverstein et al. 1963, Silverstein and Kraner 1965, Silverstein, Parshall and Uhr 1966, Silverstein and Prendergast 1970) and concluded (Silverstein and Prendergast 1970) that competence to react to different antigens is attained in a progressive and orderly manner (shown in Figure A). However, his claim that an hierarchical sequence of immunologic maturation is remarkably constant from one animal to another has not been confirmed by Fahey (1976). He found that although the onset of competence to synthesize antibody to most antigens occurred at a relatively discrete stage of development the ability of a group of foetuses to respond to ovalbumin and chicken gamma-globulin was quite variable until late in gestation. Another point which is apparent from Silverstein's study is the early age at which the first signs of immunological competence develop in the foetus. From the observation that the sheep foetus is capable of forming antibody to øx 174 at 35 days gestation, he suggested that the development of immunological competence may proceed in the absence of discernible lymphoid maturation (Silverstein and Prendergast 1970). Later work has shown that thymic lymphopoiesis commences at 36 days gestation (Jordan 1976).

Another intriguing aspect of the foetal lamb is the slight influence that foetal thymectomy has on the subsequent development of immunocompetence. Although *in utero* thymectomy did have significant effects on the development and growth of lymphoid tissues (Cole and Morris 1971a, 1973, Silverstein and Prendergast 1973), antibody responses to swine influenza, salmonella, chicken erythrocytes (Cole and Morris 1971b), ferritin, ovalbumin and bovine gamma-globulin (Silverstein and Prendergast 1973) were not affected. Skin allografts were rejected normally by lambs that had been thymectomized *in utero* (Cole and Morris 1971c, Silverstein and Prendergast 1973) and only

Figure A. The ontogeny of the immune response to certain defined antigens and the time of appearance of lymphocytes in the various lymphoid organs of foetal lamb.

from Silverstein and Prendergast (1970).


ANTIGEN:
Bacteriophage
φ X 174
Ferritin
Skin
Allografts
Hemocyanin
Ovalbumin
BIRTH
Diphtheria Toxoid
Salmonella typhosa
B.C.G.
AGE POST
CONCEPTION
(DAYS)
0
<41
56
75
120
150
>42
TIME OF
APPEARANCE
OF
LYMPHOCYTES
THYMUS
LYMPH NODES
SPLEEN
INTESTINE

delayed hypersensitivity and normal lymphocyte transfer reactions were impaired in such animals (Cole and Morris 1971c, 1971d). Whether this lack of a major effect of in utero thymectomy is due to incomplete extirpation of the thymus (because of the presence of an ectopic thymus), to the early dissemination of thymus cells to the periphery or to the thymus not having a unique role in this species is not clear.

The in vitro reactivity of foetal cells has been studied. Leino (1978) reported that PHA reactive cells were found in the liver from 38 days, the thymus from 68 days and the spleen from 90 days and that Con A reactive cells were found in liver from 38 days and in the thymus and spleen from 90 days gestation. MLC-reactive cells were found in the liver from 41 days and in thymus from 58 days gestation (Asantila and Toivanen 1976). However, immunologically specific effector T cells (as determined by the cell-mediated lympholysis test) appear not to develop until after birth (Toivanen et al. 1978).

The entire range of complement activity develops only after the 123rd day of gestation (Rice and Silverstein 1964). In summary, it appears that a major degree of immunocompetence is attained at an early stage of development of the foetal sheep.

5) Man (gestation period 280 days)

The human foetus develops immunological capability at an early stage as do the foetuses of other species with relatively long gestational periods (Solomon 1971). Small lymphocytes can be found in the peripheral blood at 7 to 8 weeks gestation (Playfair, Wolfendale and Kaye 1963). Differentiation of medulla and cortex has occurred in the thymus by the middle of 8 weeks gestation (Valdes-Dapena 1957). Thymocytes respond to PHA by the 10th week (Stites, Carr and Fudenberg 1974) and acquire the capacity to form rosettes with sheep erythrocytes by the 15th week (Wybran, Carr and Fudenberg 1972). Liver, spleen and thymus cells from 16 to 24 week old foetuses respond to and stimulate

allogeneic cells in one-way MLC (Pegrum 1971). The state of CML (cell-mediated lympholysis) activity of the human foetus is in some doubt. Toivanen et al. (1978) observed an absence of CML activity in spleen cells from 10 week old foetuses, but, recently, it was reported that peripheral blood lymphocytes collected from 18 to 22 week old foetuses can kill allogeneic cells in vitro (Rayfield, Brent and Rodeck 1980).

If foetal allograft immunity is deficient, accidental movement of maternal cells across the placenta could produce serious consequences in the foetus. Kadowaki et al. (1965) has reported XX/XY chimaerism in newborn male infants who subsequently succumbed to disease similar to GVHD. XX cells were detected only in the peripheral blood (although the yield of metaphases was poor, 27 to 40% of the countable cells had an XX constitution) but not in other tissues. However, it could be argued that congenital immunodeficiency of the foetus was primarily responsible for the chimaerism and the subsequent GVHD like symptoms. Indeed, the incidence of GVHD in the foetus or neonate following intra-uterine transfusions or exchange transfusion has been low (Hutchinson, Turner and Schlesinger 1971, Parkman et al. 1974). Fowler, Schubert and West (1960) have reported that one prematurely born infant at an estimated age of 32 weeks was fully capable of rejecting his father's skin by the 12th day. Therefore, it would be reasonable to conclude that alloreactivity develops well before this age in humans.

The presence of foetal suppressor cells has been proposed as one of the mechanisms by which the foetus evades maternal attack (Olding and Oldstone 1974, 1976, Olding, Murgita and Wigzell 1977, Oldstone, Tishon and Moretta 1977). These investigators observed that cord blood lymphocytes significantly inhibited the division of maternal cells in response to PHA and PWM (pokeweed mitogen). This suppressive activity was mediated by the liberation of a factor from newborn T cells. The extent of immunological specificity of the factor was not investigated. However, a recent report shows that the magnitude of bidirectional MLR between maternal and

newborn cells is almost the same as that between paternal cells and newborn cells or that between third-party and newborn cells or that between third-party cells and paternal cells (Moen et al. 1980). This indicated that newborn cells did not exert any detectable suppression on the division of maternal cells in MLC and hence the significance of the results of Olding's group is not clear.

Surface immunoglobulin-positive cells have been demonstrated in foetal liver at 9.5 weeks gestation (Lawton et al. 1972). Van Furth, Schuit and Hijmans (1965) detected cells producing immunoglobulins in tissues from foetuses of 20 weeks gestation. However, the possibility remains that these cells were derived from the mother. Martensson and Fudenberg (1965) detected IgG bearing Gm(a+) or Gm(b+) allotypes in cord sera from infants of Gm(a-) or Gm(b-) mothers respectively, indicating that the foetus is capable of producing immunoglobulins. Eichenwald and Shinefield (1963) detected anti-toxoplasma activity in the 19S fraction obtained from cord sera of infants with congenital toxoplasmosis but not in the 19S fraction obtained from maternal sera, indicating that IgM anti-toxoplasma antibody was produced by the infected foetuses.

Although the results cited above suggest that the repertoire of immune responses is extensive even before birth, human newborns are especially susceptible to certain types of infections. In some cases, the severity of the illness seems to depend largely on whether the causative agent of the disease stimulates production of the transmissible immunoglobulin, IgG, in the mother (Solomon 1971). Solomon speculated that the susceptibility of newborns could reflect sluggishness of the primary response with consequent failure to counteract the rapid rate of multiplication of the pathogenic organisms.

C. MATERNAL REACTIVITY

Medawar (1953) noted that Rh immunization during pregnancy occurred

much less frequently than would have been expected on purely immunological grounds and, consequently, inferred that the immunological capacity of the mother may undergo a physiological change that serves to protect the foetus from possible maternal attack. To test the suggestion that maternal reactivity is modified during pregnancy, a variety of nonspecific and specific parameters of immunity have been examined.

One aspect of maternal reactivity to have been investigated is the disease susceptibility of pregnant women. Pregnant women have been reported to be particularly susceptible to poliomyelitis (Siegel and Greenberg 1965), influenza pneumonia (Francis and Maassab 1965), hepatitis and smallpox (Beer and Billingham 1976). However, the contribution, if any, of impairment of immunological reactivity to this heightened susceptibility is completely speculative.

Significant changes occur in the lymphoid tissues during pregnancy. The human thymus involutes significantly (Nelson and Hall 1965), and a similar occurrence has been reported in mice (Persike 1940, Pepper 1961, Ito and Hoshino 1962, Maroni and De Sousa 1973, Chambers and Clarke 1979) and guinea pigs (Simmons 1964). That the extent of involution in mice is similar in response to syngeneic and allogeneic matings suggests that the phenomenon is not a response to the foreign nature of foetal tissue (Chambers and Clarke 1979). Similar involution occurs in pseudo-pregnant mice and there is a negative correlation between progesterone levels and thymic involution (Chambers and Clarke 1979). Both of these observations suggest the involvement of hormonal factors in involution. Immunization of female mice with skin grafts of paternal strain prior to mating abolished this involution in some strain combinations (Clarke 1979). Thymic involution was not observed in all strains of rats (Bauminger and Peleg 1978) indicating that it was not a prerequisite for successful pregnancy.

Decrease in the frequency of germinal centres has been observed in the pelvic (Nelson and Hall 1964) and para-aortic nodes (Nelson et al. 1973) of pregnant women. In contrast, Beer and Billingham (1972) described a significant increase in the weight of the para-aortic lymph node in pregnant Fischer rats carrying histoincompatible (Fischer x DA)F_1 hybrid foetuses. No significant degree of regional adenopathy was observed in the case of intra-strain matings. They reported that a similar increase in lymph node weight occurred when allogeneic skin grafts, lymphoid cells or spermatozoa were inoculated into the uterine lumen. This enlargement was specific in the sense that it was not incited by syngeneic grafts or tissues. Maroni and De Sousa (1973) reported that the para-aortic node became heavier in response to allogeneic (Balb/c x C3H) than to syngeneic pregnancy (Balb/c x Balb/c). However, Hetherington and Humber (1977), using several strains of mice, found no correlation between hypertrophy of the para-aortic lymph node and the extent of genetic difference between the parents. This profusion of conflicting results indicates the limitation of experiments using limited numbers of allogeneic strain mating combinations and any attempt to construct a general hypothesis from these results should be eschewed until information from matings between a large number of strain combinations becomes available. Species differences could also be crucial if the time-scale of physiological changes induced by pregnancy is such that there is insufficient time for evidence of the change to develop in those species with a shorter gestational period.

Pregnant animals are capable of producing a wide range of antibodies against foetal antigens. The production of anti-Rh antibody by Rh negative women has been known to be a consequence of maternal sensitization to foetal antigens. However, the incidence of Rh antibodies in first pregnancies is very low (reviewed by Tovey and Maroni 1976). Leucoagglutinins or cytotoxic antibodies also appear in maternal serum (Van Rood Eernisse and Leeuwen 1958, Payne and Rolfs 1958).

These antibodies can be detected in from 10 (Burke and Johansen 1974) to 15.4% (Zmijewski, Zmijewski and Huneycutt 1967) of primiparous women and the incidence tends to increase with parity (Zmijewski, Zmijewski and Huneycutt 1967, Burke and Johansen 1974). Maternal haemagglutinins and leucoagglutinins against paternal or foetal antigens can be found in multiparous mice (Herzenberg and Gonzales 1962, Kaliss and Dagg 1964, Goodlin and Herzenberg 1964). Lymphocytotoxic antibodies occur in 52% of multiparous sheep and they are produced within 2 to 4 weeks after the first pregnancy (Ford and Elves 1974). Recent studies using the horse, of which the placenta is epitheliochorial shows that in first-foal mares lymphocytotoxic antibodies are often detectable by the 60th day after conception and that they occur in approximately 90% of mares by the 3rd week postpartum (Antczak 1980).

Heslop, Krohn and Sparrow (1954) have reported that skin allografts transplanted to rabbits which are between 20 and 24 days of pregnancy survive for approximately twice the duration of allografts transplanted to recipients at other stages of pregnancy or to normal males or females. It may be relevant in this context that corticosteroid production in the pregnant rabbit is at its peak at 3 weeks and Medawar (1953) has suggested that corticosteroids might fulfil an important role in achieving 'maternal inertia' during pregnancy. Anderson (1970) exchanged skin grafts between the mother and foetus in armadillos, rats, dogs and sheep and observed an evanescent, but significantly delayed homograft reaction on the part of recipients in later stages of pregnancy. However, the interpretation of this phenomenon is difficult given the lack of information about the degree of histoincompatibility between mother and foetus in those outbred species. It could be that the prolongation of graft survival was a consequence of genetic proximity between mother and foetus (Beer and Billingham 1976).

Other researchers have certainly failed to confirm the occurrence of maternal hyporeactivity using skin grafting techniques. Foetal hind-limb

grafts were rejected by pregnant rats in a normal fashion (Woodruff 1958). Normal skin graft rejection was observed in cattle in late pregnancy (Billingham and Lampkin 1957). Skin allografts were rejected normally by multiparous A and C3H mice (Medawar and Sparrow 1956).

The only report of specific impairment of in vivo reactivity in the first pregnancy was that of Currie (1970). A_2G female mice were mated with either A_2G or CBA males. When a CBA-derived sarcoma was transplanted to these mice on the 9th day of pregnancy, it was found that the tumour grew better in A_2G females mated with CBA males than in A_2G mice mated with A_2G males or virgin A_2G mice. Although this observation has been quoted as an example of specific maternal hypo-reactivity, Currie stated that "in a first pregnancy, this enhancement is not a powerful effect and cannot explain the failure of rejection of the conceptus as an allograft" (Currie 1970).

All other observations of specific in vivo maternal hyporeactivity have been made, not in primiparous females, but in postpartum or multi-parous animals. Breyere and Barrett (1960a) have reported a significant-ly protracted survival of skin grafts of paternal strain in 'parous' female mice and this phenomenon has long been cited as a reflection of maternal hyporeactivity during pregnancy. However, the fact that the skin grafts were applied 15 days after delivery suggests that the observed changes may well have been parity- rather than pregnancy-induced. The distinction between effects of pregnancy and parturition on maternal immune reactivity becomes particularly important if any claims are to be made that changes in reactivity fulfil a function in the maintenance of pregnancy. Breyere has also reported maternal 'tolerance' in pregnant mice (Breyere and Barrett 1960b, 1960c, Breyere and Burhoe 1964, Breyere 1967, Breyere and Spiess 1973) but in all instances multiparous mice were used and so the changes may have been parity-induced. An example of a parity-induced phenomenon is Rh haemolytic disease which is rarely

observed in babies born to primiparous women and is believed to be caused by the accidental entry of foetal blood cells into the maternal circulation during labour.

In vitro assays have been used extensively in attempts to characterise the immunoregulatory processes operative in pregnancy. The PHA responsiveness of maternal lymphocytes has been reported to be depressed (Purtilo, Hallgren and Yunis 1972, Walker, Freeman and Harris 1972, Finn et al. 1972, Nelson et al. 1973, Strelkauskas, Davies and Dray 1978), unchanged (Comings 1967, Thiede, Choate and Dyre 1968, Watkins 1972, Hsu 1974, Birkeland and Kristoffersen 1977, 1980a) and increased at suboptimal mitogen doses (Carr and Stites 1972, Stites and Fudenberg 1973). MLC reactivity of maternal cells has been reported to be specifically decreased (Lewis et al. 1966), nonspecifically decreased (Jones and Curzen 1973) or unchanged (Carr, Stites and Fudenberg 1974, Birkeland and Kristoffersen 1980b, Moen et al. 1980). Finn and St Hill (1978) have reported that significant hyporeactivity of maternal cells to cells of the offspring, although not demonstrable with 1-way MLC assays, could be shown by the use of 2-way MLC. However, this claim has not been confirmed by others (Moen et al. 1980).

It will be noted that there are considerable variations between reports in the methods of assessment of lymphocyte proliferation. In earlier studies, whole blood (Comings 1967, Finn et al. 1972) or leucocytes (Thiede, Choate and Dyre 1968, Watkins 1972, Purtilo, Hallgren and Yunis 1972, Jones and Curzen 1973, Hsu 1974) were used because of the nonavailability of techniques for purifying lymphocytes, so the possibility of interference with lymphocyte reactivity by other cell types such as monocytes and neutrophils cannot be excluded. Morphological assessment of lymphocyte transformation as adopted in earlier studies (Lewis et al. 1966, Comings 1967, Finn et al. 1972) is a qualitative analysis and, as such, is unavoidably subject to observer bias.

The dose of PHA used is also an important variable since the dose range for optimal stimulation is known to be narrow. Carr, Stites and Fudenberg (1973) conducted dose/response experiments and concluded that, at optimal doses of PHA, maternal cells reacted normally. The most important variable in this type of in vitro assay may be the presence or absence of maternal serum or plasma in the culture. Since a variety of humoral factors which inhibit cellular proliferation have been reported in maternal serum and plasma (Stites et al. 1979), it is difficult to assess the intrinsic reactivity of lymphocytes if maternal plasma or serum is incorporated in the culture.

The mode of collection of samples is another important variable. Except for a few recent reports (Moen et al. 1980, Birkeland and Kristoffersen 1980a, 1980b), most studies have been carried out in a cross-sectional way by collecting samples from a group of pregnant females at different stages of pregnancy. However, the day-to-day variations inherent in all in vitro proliferation assays render the interpretation of the results from these tests difficult. In this sense, a longitudinal investigation in which each pregnant female is examined chronologically would be more informative than studies based on single-point examinations of many pregnant females. It is notable that in recent reports in which human lymphocytes were sequentially collected and cryopreserved at intervals during pregnancy, and then tested at a single time, no change in MLC reactivity of maternal cells was observed during pregnancy (Moen et al. 1980, Birkeland and Kristoffersen 1980b).

Lymphocytes collected from mice and humans during pregnancy are not cytotoxic for paternal strain cells, but can be primed to manifest cytotoxicity for the paternal cells after coculture with irradiated paternal cells (Wegmann et al. 1979, Bonnard and Lemos 1972, Granberg, Hirvonen and Toivanen 1979).

Apart from the functional assays described above, the status of

lymphocyte subpopulations in the peripheral blood have been investigated in pregnant women. Bulmer and Hancock (1977) reported a decrease in the circulating T cell levels of pregnant women but this has not been confirmed by others (Dodson et al. 1977, Birkeland and Kristoffersen 1979). It is quite conceivable that any reduction of T cells in the circulation could represent not a decrease in their absolute number _in vivo_ but rather their re-distribution or sequestration in some organs. Reports of monocyte or macrophage function during pregnancy are few. Hawes, Kemp and Jones (1980) have reported that there was an increase in the activity of monocytes in the blood of pregnant women in the second and third trimesters. These _in vitro_ studies provide little evidence for intrinsic deficiencies of those cell types which are implicated in mounting immune responses in pregnant animals.

There has been considerable discussion about the ability of hormones to act as immunosuppressants in the pregnant animal. Recent studies in humans have shown that the concentrations of a variety of gestational steroids and protein hormones in maternal serum never attain the levels required for demonstration of immunosuppression _in vitro_ and the possibility that higher levels of these factors may occur in some tissues to produce local effects has been suggested (Schiff, Mercier and Buckley 1975, Stites et al. 1979).

Daily injection of mice with human chorionic gonadotropin (HCG) has been reported to cause marked atrophy of the thymus and spleen and crude preparations of HCG inhibit human lymphocyte proliferation _in vitro_ (Jenkins et al. 1972, Adcock et al. 1973, Contractor and Davis 1973). However, recent reports have clearly established that contaminating factors rather than HCG are responsible for the immunosuppressive activities which had been reported (Morse et al. 1976, Siiteri et al. 1977, Maes and Claverie 1977). Similar allegations have been made about alpha-fetoprotein (AFP) which has been claimed to be strongly immunosuppressive in mice (Murgita

and Tomasi 1975a, 1975b, Yachnin 1976). Both highly purified rat AFP (Parmely and Thompson 1976) and human AFP (Goekin and Thompson 1977) have been reported to lack the suppressive activities described earlier. These more recent findings necessitate a complete re-evaluation of the role of other pregnancy-associated substances using purified fractions.

Hellström, Hellström and Brawn (1969) have reported the presence of a 'blocking factor' in pregnant mouse serum because serum from Balb/c females pregnant to C3H males prevented the inhibition by specifically presensitized Balb/c lymph node cells of in vitro colony formation by C3H embryonal cells. Later work suggests that this factor consisted of complexes between embryonic antigens and maternal antibody (Tamerius, Hellström and Hellström 1975), but other attempts to demonstrate the existence of 'blocking factor' have been unsuccessful (Brent et al. 1972) as also have attempts to detect circulating immune complexes in pregnancy sera (Stites et al. 1979).

It has been shown that multiparous serum can nonspecifically inhibit the MLC reactivity of lymphocytes and that this effect is mediated by IgG although no clear anti-HL-A specificities could be demonstrated (Buckley, Schiff and Amos 1972, Gatti, Yunis and Good 1973, Robert, Betuel and Revillard 1973). The suggestion has been made that these antibodies may be directed against 'Ia' like antigens (Winchester et al. 1975, Jeannet et al. 1977). Rocklin, Kitzmiller and Kaye (1979) found that more than 80% of normal pregnant women possessed blocking factors which could prevent the MIF response of maternal cells to paternal cells, whereas chronic aborters lacked these factors.

Morton reported the appearance within 24 hr of fertile mating of an early pregnancy factor (EPF) that was detectable because of its capacity to reduce the requirement for anti-lymphocyte serum (ALS) to inhibit rosette formation with sheep red blood cells by lymphocytes. EPF activity

was observed both with lymphocytes obtained from pregnant donors and with lymphocytes that had been preincubated with serum from pregnant donors. The phenomenon was observed in the mouse (Morton, Hegh and Clunie 1976), the human (Morton et al. 1977) and the sheep (Morton et al. 1979).

EPF was claimed to be immunosuppressive because of its ALS-sparing property and also on the basis of the observation that EPF prepared from sheep abolished the ability of trinitrochlorobenzene (TNCB)-sensitized mouse lymph node cells to transfer TNCB hypersensitivity into syngeneic recipients (Noonan et al. 1979). However, the use of a dubious mouse ear thickness assay to measure xenogeneic preparations of EPF necessitates further confirmation, if an immunosuppressive role for EPF is to be accepted. Thompson et al. (1980) were unable to demonstrate the alleged alteration in sensitivity of human lymphocytes to ALS during pregnancy.

Although there have been reports suggesting the presence of suppressive factors in maternal serum as discussed above, further studies are required to assess the significance of these humoral factors and their relevance, if any, for the successful completion of pregnancy.

D. TRANSMISSION OF ANTIBODIES

The placenta appears to exhibit a greater diversity of structure and function between species than any other mammalian organ (reviewed by Steven 1975). It is necessary to recognize this heterogeneity when foetal-maternal interactions are discussed. The classification of the placental types of a wide variety of species was first established on histological criteria by Grosser (Amoroso 1952) on the basis of the number of layers tissue which separated foetal from maternal blood streams (Table A). However, later studies revealed that placentae within the same general category can differ markedly from one another in fine structure. Amoroso's

Table A. Tissues separating foetal and maternal blood.

Classification (Grosser)	Maternal tissue (Uterine mucous membrane)			Foetal tissue			Gross form of placenta	Typical examples
	Endothelium	Connective tissue	Epithelium	Trophoblast	Connective tissue	Endothelium		
Epitheliochorial	+	+	+	+	+	+	Diffuse	Pig horse
Syndesmochorial	+	+	–	+	+	+	Multiplex	Sheep goat cow
Endotheliochorial	+	–	–	+	+	+	Zonary	Cat dog ferret
Haemochorial	–	–	–	+	+	+	Discoid	Man monkey bats mice insectivores

from Amoroso (1961)

work showed that the syndesmochorial placenta of sheep is in fact epitheliochorial in nature (Stevens 1975). Furthermore, in certain species, transition between different placental types can occur during development (Amoroso 1952).

It has been tempting to assume that the permeability of the placenta to antibodies may be inversely proportional to the number of layers of tissue between the maternal and foetal circulation and superficial evidence for this proposition is presented in Table B. However, Solomon (1971) refuted this hypothesis for the following reasons:

1) Electronmicroscopic studies by Amoroso (1961) have revealed that, irrespective of placental type, separation of maternal and foetal circulations is achieved only by a thin layer of cells over quite a large area.

2) Although the rabbit and guinea pig have a haemochorial placenta (like man), no maternal antibody is transmissible by this route; all antibody is transmitted to the foetus exclusively via the yolk sac.

It seems, therefore, that there is no convincing explanation at present for the complete lack of transmission of antibodies in ruminants. Possible routes for transmission of immunity can be classified as follows.

1. placental (man, monkey)
2. yolk-sac (guinea pig, rabbit)
3. placental and colostral (mouse, rat, cat, dog)
4. colostral (horse, pig and ruminants).

The transmissibility of antibodies seems to be related more to the isotype of immunoglobulin rather than to their molecular weight. In the human, IgG molecules are readily transmitted across the placenta whereas IgA, IgM and IgE molecules are not (Loke 1978). This selective transmission cannot be explained by molecular weight of the antibody class because the monomeric form of IgA has a similar molecular weight to IgG.

Table B. Superficial evidence that the thickness of the maternal-foetal placental barrier prevents transmission of maternal antibody

Species	Placental type	Number of tissue layers	Transmission of maternal antibody	
			Pre-natal	Post-natal
Horse, pig	Epitheliochorial	6	-	+++
Cow, sheep	Syndesmochorial or Epitheliochorial	6	-	+++
Dog	Endotheliochorial	4	+	++
Rat, mouse	Haemochorial	4	+	++
Rabbit, guinea pig	Haemochorial	2	+++	-
Man, monkey	Haemochorial	2	+++	-

from Solomon (1971)

It is pertinent to review briefly the consequences of transfer of maternal antibody. It has been shown that maternal sensitization to paternal histocompatibility antigens can take place in human pregnancy (van Rood, Eernisse and Leeuwen 1958). Terasaki et al. (1970) investigated the effect of maternal sensitization on the offspring and reported that women with anti HL-A antibodies gave birth to a significantly higher frequency of infants with congenital anomalies than did women who lacked antibodies. However, later reports failed to confirm this observation (Sever and Terasaki 1970, Ahrons 1971b). Attempts to demonstrate correlation between HL-A incompatibility of the parents and early spontaneous abortion have also been unsuccessful.

Rh haemolytic disease is by far the most important consequence of transfer of antibody across the human placenta. Similar diseases can be observed in ruminants as a result of colostral transfer of antibody (Caroli and Bessis 1947).

ABO incompatibility also can cause haemolytic disease of newborn but it is generally mild and rarely requires treatment (Loke 1978). Other interesting features of ABO haemolytic diseases are that it is confined to babies of group 'O' mothers and that the disease can be seen in babies of primiparous women. Loke (1978) has speculated that there may be two clinical types of ABO haemolytic disease; one which is caused by spontaneously occurring blood group IgG antibody that can affect first-born babies, and the other which is caused by IgG isoantibodies produced in response to exposure to incompatible foetal red cells as in the case of Rh haemolytic disease.

There is some evidence that maternal sensitization may suppress phenotypic expression of histocompatibility antigens of foetal cells in humans. Tiilikainen, Schröder and De La Chappelle (1974) reported a selectively decreased phenotypic expression of paternally derived antigens on foetal lymphocytes and a similar observation has been made by Dumble

et al. (1977) using a microabsorption technique. The evidence suggesting that transmitted maternal anti-HL-A antibodies were responsible for this phenotypic change includes,

(1) only the paternally derived antigens on foetal cells were decreased,

(2) expression of the paternal antigens could be restored by overnight incubation of these cells (Tiilikainen, Schröder and De la Chapelle 1974) and

(3) incubation of adult cells which were known to possess the paternal antigens with either maternal or foetal serum could produce significant reduction in the ability of the cells to express those antigens (Dumble et al. 1977).

Practically all of the IgG found in the serum of the newborn rabbit is of maternal origin (Dray 1972), having been transmitted via the yolk sac (Brambell 1970). The immunoglobulins carry genetically determined antigenic markers known as allotypes. Dray (1962) was the first to demonstrate that maternal antibodies directed against an allotype of the progeny can interfere substantially with the ability of the young to express this allotype in its serum. This suppression may persist throughout the life of the rabbits (Dray 1972). Whether a similar phenomenon can occur in other species of animals or not is an interesting question. Herzenberg et al. (1967) succeeded in inducing short-lived allotype suppression in newborn mice by raising anti-paternal allotype antibody in pregnant mice. However, using the F_1 hybrid of a particular strain combination, it was possible to induce a long-term suppression similar to that observed in the rabbit (Jacobsen and Herzenberg 1972). Lieberman and Dray (1964) suggested that a similar mechanism could affect the well-being of the foetus in view of the high incidence of maternal and foetal deaths in pregnant mice producing allo-antibodies to paternal immunoglobulin allotypes. When they immunized Balb/c females against C57BL/6

immunoglobulin and then mated them with C57BL/6 males, as many as 50% of the pregnant Balb/c mice died at term or shortly thereafter and the survival of the progeny was only 30% of control groups. In the reciprocal combination (C57BL/6 females immunized against Balb/c immunoglobulin and subsequently mated with Balb/c males), there was no maternal mortality but no live progeny were delivered. Confirmation of this report has yet to be published.

There does not appear to be any conclusive evidence that allotype suppression takes place in humans, while the presence of maternal anti-paternal allotype antibodies does not seem to be associated with any clinically obvious symptoms in the foetus or newborn (Loke 1978).

E. CELLULAR EXCHANGE BETWEEN MOTHER AND FOETUS

The possibility of spontaneous transplacental passage of cells has been extensively investigated. There have been a number of reports suggesting that the human placenta is not completely impermeable to the passage of cells in either direction (reviewed by Brambell 1970, Solomon 1971). However, all the available evidence suggests that the placenta of ruminants, and especially of sheep, is impermeable to cells and immunoglobulins (Mason, Dalling and Gordon 1930, Brambell 1970).

The presence of foetal red cells in pregnant women antepartum and postpartum has been observed frequently (Chown 1954, Woodrow et al. 1965, Sullivan and Jennings 1966, Beer 1969). Estimates of red cell traffic have suggested that the cellular elements of from 0.04 to 40.0 ml of foetal blood have been detected in the maternal circulation postpartum (Beer 1969). These calculations utilized the acid elution method of Kleihauer which takes advantage of the higher resistance of foetal compared with adult red cells to acid treatment (Kleihauer, Braun and Betke 1957). However, it has to be borne in mind when such results are

interpreted that there are some inherent errors in this technique. Possible sources of error are

(1) some foetal cells contain more haemogloblin A than haemoglobin F in late stages of gestation and so would not stain with this technique,

(2) red cells with haemoglobulin F can be found in adult life in various pathological conditions and even in some normal adults (Loke 1978).

Transfer of erythrocytes from the human mother to her foetus has been demonstrated using radio-labelled cells (Naeslund 1951, Smith et al. 1961).

The exchange of leucocytes between the male foetus and its mother has been documented on the basis of identification of Y chromosomes in metaphase spreads of cells collected from the mother and subsequently stimulated with PHA (Walkanowska, Conte and Grumbach 1969, Whang Peng et al. 1973). However, a report by Jacobs and Smith (1969) that '46XY' cells can be found in normal non-pregnant women necessitates caution in the interpretation of these karyotypic studies, especially as PHA stimulates only a subpopulation of lymphocytes. The use of quinacrine mustard to visualize Y chromatin in interphasenuclei (Pearson, Bobrow and Vosa 1970) has obviated the necessity for cultivation of cells and removed the complications of stimulating only a subpopulation of cells. Schröder and De la Chappelle (1972) claimed that Y bodies were found in lymphocytes collected from some pregnant women. Although a strong correlation was found between the presence of Y-body containing cells and the carrying of a male foetus, the discordant observation that some pregnant women who had been identified as possessing 'male' cells eventually produced female offspring. Herzenberg et al. (1979) used a fluorescence activated cell sorter (FACS) to select foetal cells from maternal blood after its treatment with anti-paternal antiserum. The

cells selected in this way were then scored for content of Y chromatin using quinacrine mustard. They were able to identify in maternal blood samples those cells which possessed both a Y fluorescent body and paternal antigens. No 'false negatives' were observed. However, as the previous history of the mothers in this study was not known, the possibility exists that 'male' cells were carried over from a previous pregnancy.

Passage of leucocytes from mother to foetus has been documented by Desai and Cregar (1963). Two cases of XX/XY chimaerism in newborn male infants (Kadowaki et al. 1965, Githens et al. 1969) may be pertinent. The clinical symptoms observed in these infants, namely overwhelming infection, diarrhoea and skin rash, were reminiscent of GVHD.

In mice, the T6 chromosome marker has been used to identify maternal-foetal leucocyte traffic. Tuffrey, Bishun and Barns (1962) transferred blastocysts from CFW mice which did not possess T6 markers into the uterus of CBA/T6T6 foster mothers and claimed that from 1.2 to 28.6% of the cells in the lymphomyeloid complex of these transferred CFW offspring possessed a T6 chromosome. However, attempts by other workers to confirm this observation have been unsuccessful (Beer and Billingham 1976). Trentin, Gallagher and Priest (1977) mated CBA/T6T6 females with A or C3H mice and investigated the karyotype of the cells in F_1 hybrid animals 14 or 21 days after birth. All cells were found to be of F_1 type containing only one T6 marker chromosome.

A striking feature of the reports of exchange of cells between foetus and mother is the frequency with which discordant observations have been made. The most frequent objection to positive reports of the appearance of presumptive foetal cells in the mother has arisen from suggestions that similar cells could be demonstrated in non-pregnant adults. One consequence of the latter type of report is that, in seeking to demonstrate foetal cells in the circulation of a pregnant female, one may be dealing

with quantitative rather than qualitative differences from non-pregnant controls. Furthermore, the restriction of some of the reported 'transfer' phenomena to certain combinations of mating strains raises doubts about the general applicability of the results even if the interpretation of the observations is accepted.

Although I have referred indirectly to some of the reported consequences of foetal-maternal interactions in discussing transplacental passage of cells it is pertinent at this stage to note reports which deal primarily with such consequences. A number of attempts have been made without much success to interfere with pregnancy by prior immunization of the mother against paternal antigens. Mitchison (1953) reported normal reproductive performance on the part of female mice immunized to paternal antigens. Woodruff (1958) removed foetal tissue from one uterine horn and transplanted it to an ectopic site. Although the transplanted tissue was rejected by the mother, development of the foetus in the other horn was unaffected. Lanman, Herod and Fikrig (1964), using rabbit embryo transfer, established a situation in which the surrogate mother was immune to both parents of the offspring it carried. Despite this, abnormal foetal wastage was not observed. Immunization of ewes against the sire had no effect on fertility (Boshier and Moriarty 1970). Results of this type have often been interpreted as evidence for the effectiveness of the placental barrier. However, there have been sporadic conflicting results.

Breyere and Sprenger (1969) demonstrated that specific maternal immunity to paternal antigen could impair the fertility of the mother. They used paternal strain tumour allografts to immunize mothers. However, Parmiani and Della Porta (1973) reported that immunization of the mother with a syngeneic tumour could also be harmful to the progeny. Hamilton et al. (1979) observed decreased maternal fertility following immunization with syngeneic teratocarcinoma cells. These appear to suggest that immunity against foetal rather than histocompatibility antigens may impair

the maternal reproductive performance.

Runting was observed in rat offspring following transfer to pregnant females of syngeneic lymphocytes sensitized against paternal antigens and also in response to sensitization of females with paternal skin grafts before mating (Beer and Billingham 1972). This work did not establish whether maternal lymphocytes gained access to the foetus via the placenta and later study suggested that transfer of cells to the neonate via milk was likely to have occurred (Head and Beer 1978). Using a selected combination of Ag-B incompatible rats Milgrom, Comini-Andrada and Chaudrhy (1977) observed a high level of foetal and neonatal mortality in F_1 hybrid rats derived from mothers which had been extensively immunized against paternal skin grafts.

Uphoff (1977) reported a high incidence of neonatal runting in certain mating combinations of mouse strains. This runting was similar to that observed in rats by Palm (1969, 1970) and appeared after two weeks of life. The observation that fostering of newborns to appropriate mothers immediately after birth prevented the development of GVHD led her to believe that maternal cells in milk were responsible for the GVHD.

Exposure to foreign antigens at an early stage of foetal development has been shown to induce immunological tolerance in certain species. It is questionable whether offspring can spontaneously become tolerant of maternal antigens as a consequence of exposure to maternal cells during foetal life. If this phenomenon were to occur, it would be expected to be most prominent in those species which possess 'permeable' placentae. Billingham, Palm and Silvers (1965) used the Ag-B incompatible Lewis and BN strains and prepared (BN x Lewis)F_1 hybrid animals which had received transplanted Lewis ovaries after ovariectomy. These F_1 hybrids were mated with Lewis males so that Lewis foetuses could be exposed to the F_1 hybrid milieu, which included BN antigens. Despite this exposure, none of the offspring were hyporeactive to BN skin grafts. Peer (1958) claimed that skin grafts

transferred from mother to child survived significantly longer than those from father to child but this work has never been confirmed (Beer and Billingham 1976). Uphoff employed some ingenious techniques to investigate the possibility of maternally induced tolerance (1970a, 1970b, 1973, 1974). If some strains of F_1 hybrid animals receive parental strain bone marrow after lethal irradiation, a high incidence of GVHD is observed. Using this system, Uphoff (1970a) observed that GVHD was less severe if the donor strain of marrow was syngeneic with the maternal strain of the hybrid and raised the possibility of a qualitative difference of antigenicity between reciprocal hybrids. She speculated that this result reflected maternal modification of paternally derived antigens in the F_1 hybrid offspring as a result of exposure in utero. To investigate maternally induced alterations, she established two mouse substrains (designated ova-transfer and foster-nursed substrains). Ova-transfer substrains were produced by transferring homozygous embryos from a female mouse of one genotype to a surrogate mother of different genotype. Subsequently, brothers and sisters from the transferred offspring were mated to produce the derived substrain. Foster-nursed substrains were produced by removing full-term foetuses by caesarian section and foster-nursing them onto allogeneic mice followed by inbreeding of these offspring. These substrains were used as bone marrow donors to elicit fatal GVHD in lethally irradiated recipients or as recipients of allogeneic bone marrow following lethal irradiation. From these studies Uphoff (1973, 1974) obtained evidence that the maternal environment had influenced the phenotypic expression of both antigenicity and immunological responsiveness in the progeny and that these alterations were permanent and vertically transmissable from mother to offspring for many generations. This change in antigenicity was detectable by bone marrow transfer but not by reciprocal skin grafts between substrains. Although these results have not yet been confirmed by others, the proposition is stimulating particularly considering the potential impact

of this mechansim on evolution.

F. POSSIBLE IMPORTANCE OF FOETAL-MATERNAL IMMUNOLOGICAL INTERACTIONS

Recent studies suggest that histocompatibility gene polymorphism is a ubiquitous finding among mammals (Götze 1977). Medawar was one of the first to address himself to the possible advantages for the species of such polymorphism. "Although there are no factual grounds for supposing that antigenic diversity is anything but an unfortunate consequence of constitutional differences between individuals of a species, yet one is under some obligation to rack one's brains for evidence of any good it might conceivably do. Only thus can antigenic polymorphism be made genetically respectable" (Medawar 1953).

It is a longstanding observation in animal husbandry that hybridization of two animals with different genetic backgrounds tends to produce offspring superior in quality to those produced by matings between genetically similar parents. Although the mechanism by which this improvement is produced is not known, it has generally been considered to result entirely from heterosis. One of the first indications that factors other than the genetic constitution of the offspring could contribute was the observation of Billington(1964), using 2 histoincompatible strains of mice (C57BL ($H-2^b$) and A_2G ($H-2^a$)), that the placentae of the hybrid foetuses are larger than those of the pure-bred foetuses in the mothers of both strains. He claimed, on the basis of embryo transfer, that this increase in placental size could not be ascribed solely to genetic heterosis. A_2G embryos produced heavier placentae than did C57 embryos when both were transferred to C57BL surrogate mothers inferring that antigenic disparity between mother and foetus had influenced placental size (however, the placentae of A_2G foetuses in both A_2G mothers and C57BL surrogate mothers were heavier than those of (C57BL x A_2G)F_1 hybrid foetuses and this led

McLaren (1975) to challenge Billington's inference; see below). James (1965, 1967) confirmed Billington's observation and also showed that maternal immunological status with regard to paternal antigens further affected placental weight in inter-strain matings. If the mother had been immunized against paternal antigens, the placentae were heavier, whereas if the mother had been rendered tolerant of paternal antigens, placentae were smaller than those produced after mating of an immunologically normal female. Beer, Scott and Billingham (1975) using mice, rats and hamsters, confirmed Billington's observation on increased placental weight in histoincompatible matings and claimed further that litter size also increased in inter-strain pregnancy. Furthermore, Billington (1965) reported that antigenic disparity with the recipient led to increased trophoblast proliferation after intra-testicular transplantation.

Based on the observations of Billington (1964) and of James (1965, 1967) and on Medawar's postulate (1953), Clarke and Kirby (1966) put forward a novel hypothesis that an immunological interaction between a mother and its histoincompatible foetuses might favour the survival of the offspring and thereby contribute to the maintenance of histocompatibility polymorphisms. This recognition of foetal-maternal interaction as a selective influence favouring a balanced polymorphism of histocompatibility antigens has stimulated further experiments and some unwarranted speculations.

Michie and Anderson (1966) reported the failure of brother-sister mating of Wistar rats for more than 70 generations to eliminate heterozygosity at histocompatibility loci, and suggested that this was indicative of selection against homozygous animals. Palm's observations were in accordance with this notion (1969, 1970). She observed an excess of Ag-B heterozygotes among progeny of backcross matings produced from the Ag-B histoincompatible BN and DA strains (1969). The excess was most commonly observed in male offspring. An unusual feature of this finding was that an excess of the Ag-B heterozygous progeny was observed only when the mother was from the

inbred strain. In the reciprocal backcrosses of F_1 hybrid females to inbred males normal segregation ratios were observed. Detailed study further disclosed that aberrant ratios in offspring resulted from pre-weaning runting which resembled GVHD clinically and histologically (Palm 1970). These observations together with the association between increased severity and early onset of the wasting syndrome and multi-parity led her to conclude that wasting was immunologically determined. With further confirmation of the apparent sex-related pressures, i.e., greater vulnerability of male homozygotes, a suggestion was made that the better survival of male heterozygotes might be part of a self-perpetuating effect. If a sufficient number of female homozygotes were left relative to the excess number of male heterozygotes, at each generation repetition of this sex-related pressure in each generation should maintain an Ag-B heterozygote excess among the reproductive population. This is a major requirement for maintenance of a balanced polymorphism (Palm 1974).

The occurrence of a similar wasting syndrome in the neonatal period was demonstrated in rats by Beer and Billingham (1972). They immunized the mother against paternal antigens and observed runting in the offspring provided that immunization preceded mating by 10 days. Preliminary local sensitization of the female against paternal antigens via the uterine horn, however, was found to result in larger number of foetuses (Beer and Billingham 1977). Further evidence for a beneficial effect of antigenic disparity and maternal immunization on reproductive performance can be found in the literature. Lappé and Schalk (1971) reported that repeated immunization of the female against the H-Y antigen following splenectomy resulted in a significant increase in the sex ratio of the offspring without affecting litter size. No discernible effect of anti-male immunization on sex ratio was observed in non-splenectomized animals. They postulated that a maternal cellular response against the H-Y antigen exerted a positive selective force on the sex ratio and that this selective advantage was

neutralized by 'blocking factors' secreted by spleen.

Interpretation of the preceding experiments has been complicated by subsequent reports which have provided conflicting observations. Hetherington (1971) was unable to confirm Billington's observation (1964) on the effect of antigenic disparity of placental size using inbred JU, CBA and C57BL strains of mice. Similarly, Finkel and Lilly (1971), using allogeneic and congenic strains of mice, differing at the H-2 locus, and Hetherington (1973) using congenic strains differing at the H-2 and H-3, failed to confirm Billington's observation (1964). McLaren (1975) criticised the interpretation of Billington's data (1964) on embryo transfer experiments in which A_2G embryos were found to produce heavier placentae than C57BL embryos when both were transferred to C57BL surrogate mothers in the light of the finding of Hetherington (1971) that the genotype of the embryo influences placental size, i.e., A_2G embryos have larger placentae than C57 embryos for genetic reasons.

Clarke (1971) was unable to repeat James' experiments on a 'tolerant' mother (1965, 1967) because attempts to induce tolerance in C57BL by neonatal injection of A_2G lymphocytes were hampered by a high incidence of GVHD but she did not observe any change of placental weight in C57BL mothers immune to A_2G, which is not in accordance with the results of James (1965, 1967).

Jones (1968) reported that disparity with regard to ABO blood types was associated with <u>smaller</u> placentae. However, later reports have not confirmed this observation (Toivanen and Hirvonen 1970).

Billington's observation of superior trophoblast growth following transfer of ectoplacental cones into the testicular sac of allogeneic recipients (1965) was not confirmed by Clarke (1969). Koren, Abram and Behrman (1968) also failed to detect a relationship between antigenic disparity and trophoblast growth following transfers of trophoblast to the mouse kidney. Although a report of Hancock, McGovern and Stamp (1968),

that the trophoblast of hybrids between goat and sheep shows much more active invasive growth than does that of the goat in the goat uterus, has been cited as possible evidence for involvement of immunological factors in hybrid vigour (Beer and Billingham 1976), another report on trophoblast growth in interspecific hybrids (Rogers and Dawson 1970) raises doubt about the immunological nature of the previous findings. Rogers and Dawson crossed two species of Peromyscus which had placentae of similar weight and they found that the placentae were smaller in one cross, and much heavier in the reciprocal cross, than those observed in intraspecific matings, indicating that factors other than antigenic disparity were influencing the placental size.

On the basis of the hypothesis of Clarke and Kirby (1966) and related experimental data and further supported by a recent report that there is a higher-than-average incidence of genetic compatibility between couples with unexplained infertility (Komlos et al. 1977), a clinical trial is under way in which hitherto infertile women will be immunized against their husband's lymphocytes (Beer 1980, personal communication). From the data reviewed, it would seem to be reasonable to suspend assessment of the hypothesis of Clarke and Kirby (1966) until further experimental data is available.

Aims of the present investigation

The primary aim of the present study was to detect any modifications in the cellular immunological responsiveness to paternal and foetal antigens of the ewe during a first pregnancy. Assessment of maternal responsiveness was to be based on mixed lymphocyte culture and blastogenic response to non-specific mitogens. The lymphocytes to be used were cryopreserved soon after collection to permit simultaneous assay of all samples collected from an individual ewe and thereby minimize the day-to-day variation inherent in *in vitro* lymphocyte proliferative assays. It was hoped that examination of the immunological responsiveness of primigravid ewes would clarify the effects of pregnancy *per se* (rather than parturition-related

effects) and their relevance, if any, for the successful completion of pregnancy.

A second aim was to test the capacity of ewe and foetal lamb to respond to deliberate challenge with lymphocytes from the other. It was anticipated that cannulation of either an efferent lymphatic from the maternal regional lymph node draining the site of challenge with foetal lymphocytes or, of the thoracic duct of foetal lambs challenged with maternal lymphocytes, by allowing continuous monitoring of cellular and humoral events in the efferent lymph, would permit a detailed scrutiny of the homeostatic mechanism operating between mother and foetus.

MATERIALS AND METHODS

A. EXPERIMENTAL ANIMALS

Virgin Merino and crossbred ewes were mated with either Merino or Merino-Border Leicester crossbred rams. The rams were fitted with marking harness and the date of mating (day 0) was recorded as the day when a ewe was found to have been marked (Radford, Watson and Wood, 1960). Pregnant ewes grazed outdoors and received a dietary supplement of lucerne hay. Before and after operation, they were housed in metabolism cages and fed lucerne chaff ad libitum together with a supplement of grain oats.

B. SURGICAL PROCEDURES

1) Preoperative Procedures and Anaesthesia

All animals were deprived of feed for the preceding night. Anaesthesia was induced with 5% Pentothal (Abbott Labs.) (usually 12-16 ml were required). Following intubation with a cuffed endotracheal tube, anaesthesia was maintained on the closed circuit of a Boyle's apparatus which administered 1% fluothane (Halothane, ICI) and 1.5ℓ oxygen/min (Medical grade CIG).

The surgical area was closely clipped and scrubbed thoroughly with 1.0% Hibitiane solution (ICI) and the whole animal was covered with sterile drapes leaving only the operation site exposed.

2) Cannulation of the Efferent Duct of the Popliteal Lymph Node

This was performed by the technique described by Hall (1964) and Hall and Morris (1962). The popliteal lymph node is located in the popliteal fossa between the biceps femoris and semimembranous muscle, closely related to the vena saphena lateralis. A 15 cm long incision was made on the postero-lateral aspect of the thigh parallel to the posterior margin of the leg starting 2 to 3 cm below the sciatic tuberosity. The muscle junction between the biceps femoris and semimembranous muscle was split longitudinally and the popliteal fossa was exposed by ligating and dividing a pair of

intramuscular blood vessels followed by full separation of the muscles with a self-retaining retractor. The efferent lymphatic of the popliteal node was closely related to the vena saphena lateralis and was exposed easily with blunt dissection amongst the adipose tissue in the fossa. The duct was ligated and dissected free of connective tissues. When multiple lymphatics were found, all of them were ligated and one of the larger ducts was chosen for cannulation. The lymphatic was incised with iridectomy scissors and a clear vinyl cannula (Dural Plastics, N.S.W.) of appropriate size (normally SV45 (ID 0.58 mm, OD 0.96 mm) or SV55 (ID 0.80 mm, OD 1.20 mm) which had been led into the fossa through a stab wound situated anterior to the upper limit of the skin incision was introduced in a retrogade direction. If a valve hindered introduction of the cannula, a small aneurysm needle was inserted into the lumen of the duct to destroy the valve. The cannula was secured with two 2/0 silk ties. As soon as a satisfactory flow of lymph was established, the wound was dusted with Crystapen (Glaxo) and the fascia was sutured, followed by closure of the skin with Michel clips. Lymph from the cannula was collected into 250 ml sterile plastic bottles each containing approximately 100 units of powdered Heparin (CSL). Each bottle was tied to a holder secured on the antero-dorsal part of the thigh near the skin incision.

3) <u>Cannulation of the Efferent Lymphatic Duct from the Prefemoral Node</u>

This was performed using the technique described by Hall (1967) and by Cole (1969).

The efferent lymphatic was approached by a vertical incision made parallel but slightly anterior to the cranial border of the tensor fascia lata. The muscle was retracted to expose from 1 to 3 lymphatics draining dorsally under the edge of the tensor fascia lata muscle. The lymphatics were closely associated with the blood vessels supplying the prefemoral node. Cannulation was performed as described for the popliteal duct.

4) Collection of Bone Marrow Cells from the Ewe

The ewe was anaesthetized as described before and the pelvic and femoral areas were clipped and disinfected. A sterile aspiration needle was introduced into the medullary cavity of the antero-dorsal angle of the iliac crest and marrow was aspirated into a plastic syringe fitted to the needle. The marrow fluid was discharged from the syringe into Hanks' BSS plus 0.02% EDTA. This procedure was repeated until enough bone marrow cells were collected. Cells were washed twice with cold Hanks' BSS and the number of viable mononuclear cells were counted under the microscope.

5) Collection of Lymph Node Cells from the Ewe

The prefemoral lymph node was exposed by the method described before for cannulation of the efferent duct of the prefemoral node. The node was resected aseptically and cut into small pieces in a Petri dish with a scalpel blade. A single cell suspension was prepared under sterile conditions by pressing the small pieces through a steel mesh into cold Hanks' BSS and aggregates of cells were disrupted by aspiration and expulsion through a 19- or 21-gauge needle. Viability of cells was determined by trypan blue exclusion.

6) Cannulation of the Thoracic Duct of Foetal Lambs

Foetal lambs were exposed by the technique of Smeaton, Cole, Simpson-Morgan and Morris (1969) and Cole and Morris (1971a). The ewe was placed in the lithotomy position and the surgical area was disinfected as described before and draped. The abdomen was entered through the mid-line or on either side of the subcutaneous main mammary vein. The pregnant uterus was exteriorized and positioned on the belly of the ewe so as not to cause torsion of the uterine vessels. The uterus was immediately covered with a thin polythene bag to enclose it completely and retain moisture during the surgery. Thoracic duct cannulation of the foetal lamb was

performed by the technique described by Pearson, Simpson-Morgan and Morris (1976).

With the foetus on its left side within the uterus, the plastic bag and the uterus were incised with the aid of electrocautery over the area where the amnion and allantois were fused, great care being taken to avoid any major blood vessels or cotyledons. The fused foetal membranes were cut with scissors and the cut edges of the membranes were secured to the uterine wall with haemostats. The amniotic fluid was usually removed and stored in a sterile flask containing heparin and antibiotics at 37°C during the operation. The thoracic cavity was entered by removing the 7th or 8th rib on the right side. At this stage, the foetal membranes were clamped together with foetal skin, connective tissues and the uterine wall with Allis forceps. The costal pleura was then incised and the diaphragmatic lobe of the right lung was packed off to expose the thoracic aorta. The thoracic duct was usually found lying beneath the parietal pleura on the dorso-lateral aspect of the thoracic aorta. When the duct was found to be transposed with the hemiazygous vein, it was necessary to approach the duct via ventral or dorsal aspects of the aorta. The duct was freed from surrounding connective tissues and ligated with a 4/0 silk suture as far cranially as possible. A second 4/0 silk suture was passed around the duct about 1 cm caudal to the first tie. One end of a 3 metre long polyvinyl tubing (SV35; ID 0.40 mm, OD 0.80 mm or SV45; ID 0.58 mm, OD 0.96 mm, Dural Plastics) filled with a heparin-saline solution, was introduced into the thoracic cavity of the foetus through the incision. The duct was incised with iridectomy scissors and cannulated. Two anchoring sutures were placed around the cannula after it had been correctly aligned. The cannula brought out through the incision was secured to the skin and the chest wall of the foetus was reconstituted with silk sutures. A short length of the cannula was left inside the amniotic cavity. The amniotic fluid was replaced and the foetal membranes

were closed, together with the uterine wall, with a series of purse-string ligatures to produce a leak-proof seal. Continuous inverting mattress sutures were then placed in the uterine walls to prevent adhesions of the uterus to the bowel or abdominal wall. The cannula was led to the outside by passing it through a stab wound made on the right flank of the ewe. A loose coil of the tubing was left within both the uterus and the abdominal cavity of the ewe to allow for any movements of the foetus or the uterus. The abdominal incision was closed with silk sutures. Lymph was collected into sterile plastic bottles, containing approximately 100 units of powdered heparin, secured to the flank of the ewe so as to be below the level of the foetus when the ewe was lying down. After foetal surgery, daily injections of 8 ml of Strepcin (V.R. Labs) were given to the ewe for 3 days.

7) Cannulation of the Foetal Jugular Vein

The jugular vein of the foetus was cannulated with polyvinyl tubing (SV55; ID 0.8 mm, OD 1.20 mm, Dural Plastics). The cannula was secured in the vein with two silk ligatures and anchored to the foetus with a further 3 to 4 ligatures. The cannula was led out as described for thoracic duct cannulation, filled with heparinized saline (50 U/ml) and sealed by a plastic stopper connected to a 3-way valve. The cannula with the 3-way valve and a stopper was secured with a silk ligature to the wool on the back of the ewe.

8) Injection of Cells into the Foetal Lamb

i) Young foetuses (46-73 days gestation)

The uterus was exteriorized and covered with a thin polythene bag as previously described. The uterine wall was incised with the aid of electrocautery keeping the foetal membranes intact. In some cases when bone marrow cells were to be injected the foetal membranes were also incised. The foetus was located by transillumination through this window of foetal membranes and manipulated into a position underlying the window.

Allogeneic or maternal lymphoid cells were injected intraperitoneally into the foetus by inserting a 28 G- needle through the uterine wall and directly into the peritoneal cavity. After the injection was given, the inoculum was observed to disperse in the peritoneal cavity. The uterine wall was closed by a series of purse-string ligatures with 5.0 (Metric) silk thread. The abdomen was closed as described before. Following the injection, the ewes were kept under constant observation in pens for 1 week and grazed outdoors afterwards.

ii) Older foetuses (110-134 days gestation)

The jugular vein of the foetus was cannulated as described above and maternal cells were injected through this cannula. Maternal cells (7.5 x 10^7 - 6.0 x 10^9) were washed three times with Hanks' BSS and resuspended in 5 - 10 ml of Hanks' BSS before being injected into foetus. Injection was performed very slowly (5 to 10 min).

9) Skin Grafting

Skin grafting was performed according to the technique of Hall (1967). Full thickness donor skin grafts measuring 2.0 x 2.5 cm taken from the flank of the foetus during the course of foetal cannulation were dissected free of underlying fat and panniculus muscle. Grafts were transplanted to full thickness beds on the lower leg and sutured in place with 5/0 silk. Two layers of paraffin gauze dressings (Jelonet, Smith and Nephew Pty Ltd) were applied to the grafts and a sterile gauze pad was secured with Elastoplast (Smith and Nephew Pty Ltd) to provide moderate pressure to the area and prevent dehydration. Grafts were inspected after they had been in place for 5 days. Skin grafts were considered to have been rejected when the graft surface became dehydrated and developed a blue to purple colouration. Autografts were taken from the bare area of skin in the inquinal region and transplanted onto the lower leg.

C. PROCEDURES FOR NORMAL LYMPHOCYTE TRANSFER

The popliteal lymph node was challenged in vivo with foetal, paternal or unrelated lymphocytes. Preparatory techniques for these cells have been described in preceding sections. All cells were harvested and washed 3 times with cold Hanks' BSS and suspended in 0.5 ml of Hanks' BSS for injection. These cells were injected subcutaneously into multiple sites in the lower leg in which a popliteal efferent lymphatic fistula had been established.

The normal lymphocyte transfer (NLT) reaction was elicited by intradermal injection of 5 x 10^6 allogeneic efferent lymphatic lymphocytes in 0.1 ml Hanks' BSS into the wool-free medial aspect of the recipient sheep's thigh. Skin thickness was measured with 'Schnelltaster' skin calipers.

D. SEPARATION OF PERIPHERAL BLOOD LYMPHOCYTES

1) Peripheral Blood Lymphocytes from Ewes

Jugular venous blood was withdrawn into heparinized syringes. After centrifugation of the blood, the buffy coat was collected and layered onto a Ficoll-Isopaque solution (specific gravity 1.077). This was then centrifuged at 500 G for 40 min at room temperature. Cells at the interface were collected and washed twice in Hanks' BSS containing 0.75 mg/ml disodium-EDTA. The cells were washed twice more in cold Hanks' BSS.

To collect blood from the uterine vein of pregnant ewes, a midline abdominal incision was made and the uterus and adnexae were exteriorized. Blood was withdrawn from a uterine vein, via a polythene cannula, into a heparinized syringe and lymphocytes were separated as described above.

2) Peripheral Blood Lymphocytes from Foetuses

Foetal peripheral blood lymphocytes were separated by means of Ficoll-

Isopaque isopycnic centrifugation as described above and were subsequently treated with warm Tris-NH_4Cl solution to remove contaminating red blood cells. Tris-NH_4Cl solution was prepared by adding 9 vol.of 0.83% aqueous ammonium chloride to 1 vol.of Tris-buffer (20.594 g Tris base/liter, adjusted to pH 7.65 with HCl). The pH of the mixture was finally adjusted to 7.2 (Boyle 1968). Foetal cells were centrifuged and Tris-NH_4Cl solution which had been prewarmed to 37°C was added to the cell pellet. Foetal cells were resuspended and immediately re-centrifuged at 400 G for 5 min and washed twice more with Hanks' BSS.

E. FREEZING AND THAWING OF CELLS

1) Cryopreservation of Cells

Cells were suspended in Dulbecco's modified Eagle's medium (GIBCO) containing 20% foetal calf serum (Flow Labs), 10% dimethylsulphoxide (DMSO) (Mallinekrodt), 0.075% disodium EDTA and antibiotics. Two ml portions of the cell suspension were dispensed into sterile plastic vials (Sterilin, Teddington, U.K.) and frozen using a Linde BF-6 controlled rate freezer (Union Carbide, Ind., U.S.A.) at a rate of -1°C/min from 4°C to -20°C, at -2°C/min from -20 to -40°C, and at -3 to -5°C/min to -100°C and then transferred to the vapour phase of a liquid nitrogen storage container.

2) Thawing of Cells

This was done by a modification of the method of Holden et al. (1976). Frozen cells were thawed rapidly by shaking in hot water (45-50°C) and the content of each vial was transferred into a glass test tube where a dilution procedure to remove DMSO was immediately initiated as follows: 0.1 ml of Hanks' BSS containing 10% foetal calf serum (dilution medium) was added initially and this addition was repeated every 1 min, doubling the added volume each time until 5 ml was achieved. After a 20 min pause, the dilution was resumed by successive addition of 1 ml portions until the

volume was 10 ml. The cells were then spun at 150 G for 8 min at room temperature and washed twice very gently with the dilution medium.

F. TISSUE CULTURE TECHNIQUES

1) Mixed lymphocyte culture

Lymphocytes were prepared as described above and suspended in a complete culture medium consisting of Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum (Flow Labs), 10 mM HEPES (Sigma), 10^{-4} M 2-mercaptoethanol, 100 U/ml of penicillin, 100 μg/ml of streptomycin and 100 μg/ml of neomycin (complete medium). Cells to be used as stimulators were either treated with Mitomycin C (Sigma) for 30 min at $37^{o}C$ followed by washing three times with Hanks' BSS or exposed to 1500 rad gamma irradiation from a ^{60}Co source. Cell concentrations were adjusted to 5×10^6/ml for fresh cells and 6×10^6/ml for frozen-thawed cells and 0.1 ml suspensions of each of the stimulator and responder cells were mixed in a Linbro microplate with flat-bottom wells.

2) Concanavalin A (Con A) and Lipopolysaccharide (LPS) Mitogenic Responses

Con A (Sigma) and lipopolysaccharide B, E. coli 0128:B12 (Difco) were employed as mitogens to test the nonspecific immune reactivity of lymphocytes *in vitro*. Cultures were initiated by the addition of 5×10^5 fresh cells or 6×10^5 frozen-thawed cells in 0.2 ml of complete culture medium to 0.02 ml of solutions of either Con A or LPS in flat-bottomed Linbro microplates.

3) Addition of Serum or Plasma to Cell Cultures

In some experiments, 20 μl of either heat-inactivated serum or plasma was added to the culture to test its effect on lymphocyte reactivity. Preliminary experiments showed that pregnant plasma, when inhibitory, exerted its maximum effect at 10% final concentration in culture.

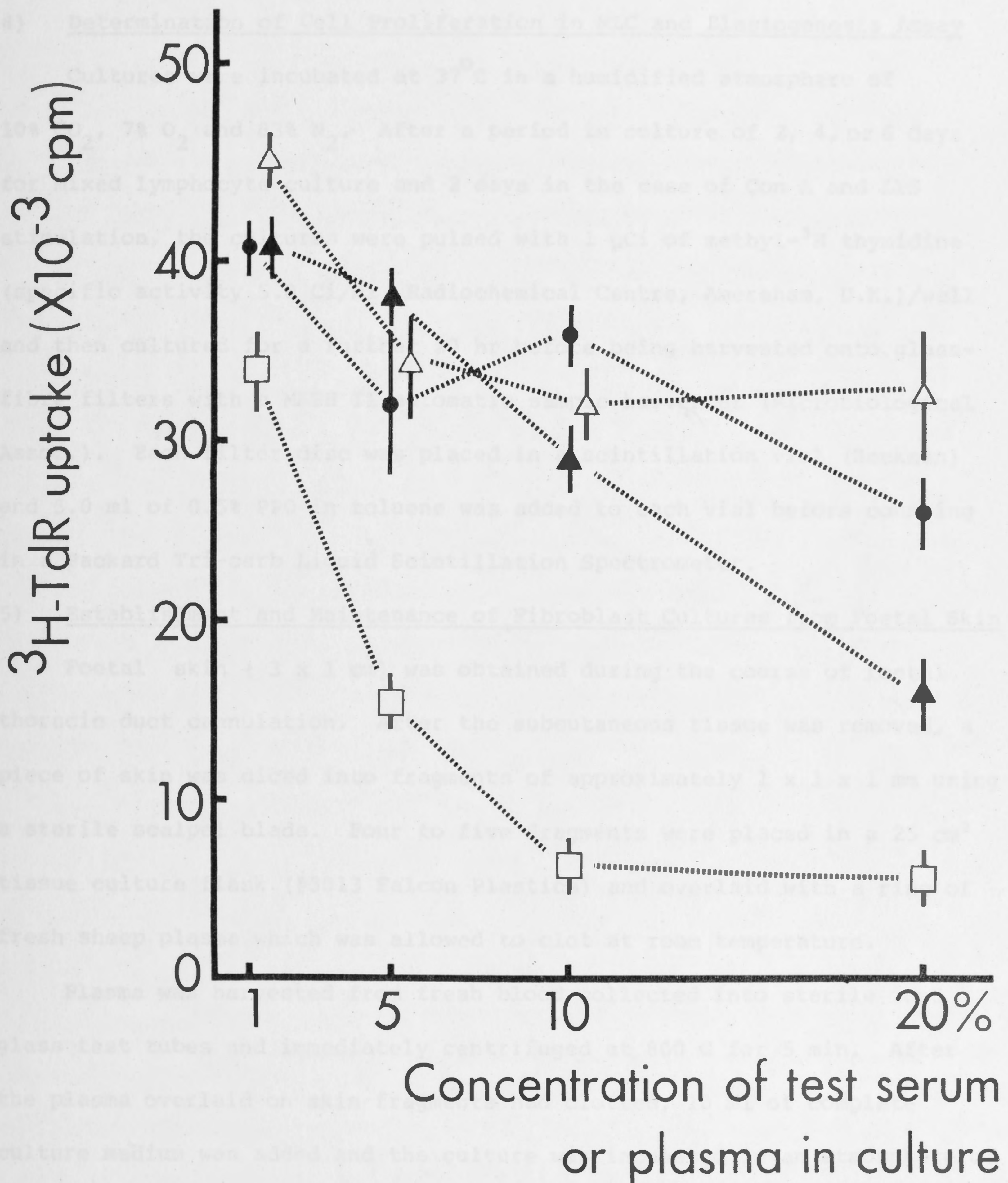
Normal plasma or serum produced a comparatively smaller inhibitory effect at this concentration (Figure B).

Figure B. Effect of serial dulutions of serum and plasma on MLC.

Sheep lymphocytes were cultured with allogeneic lymphocytes in the presence of additional

foetal calf serum (△),
normal serum (●),
normal plasma (▲),
pregnant plasma (□).

Normal plasma and serum were obtained from a virgin ewe and pregnant plasma was collected from a primigravid ewe,129 days pregnant. Cultures were harvested on day 5 and results are expressed as mean cpm ± S.E. of triplicate cultures.



3H-TdR uptake (X10-3 cpm)
50
40
30
20
10
0
1
5
10
20%
Concentration of test serum
or plasma in culture

Although heparin has been reported to be inhibitory for MLC (Currie 1967), the residual concentration of heparin, derived from plasma, in cultures did not exceed 2.5 U/ml and preliminary experiment showed that any inhibitory activity of heparin at this concentration was minimal (Figure C).

4) Determination of Cell Proliferation in MLC and Blastogenesis Assay

Cultures were incubated at $37^{o}C$ in a humidified atmosphere of 10% CO_2, 7% O_2 and 83% N_2. After a period in culture of 2, 4, or 6 days for mixed lymphocyte culture and 2 days in the case of Con A and LPS stimulation, the cultures were pulsed with 1 μCi of methyl-^{3}H thymidine (specific activity 5.0 Ci/ml, Radiochemical Centre, Amersham, U.K.)/well and then cultured for a further 20 hr before being harvested onto glass-fibre filters with a MASH II automatic sample harvester (Microbiological Assoc.). Each filter disc was placed in a scintillation vial (Beckman) and 5.0 ml of 0.5% PPO in toluene was added to each vial before counting in a Packard Tri-carb Liquid Scintillation Spectrometer.

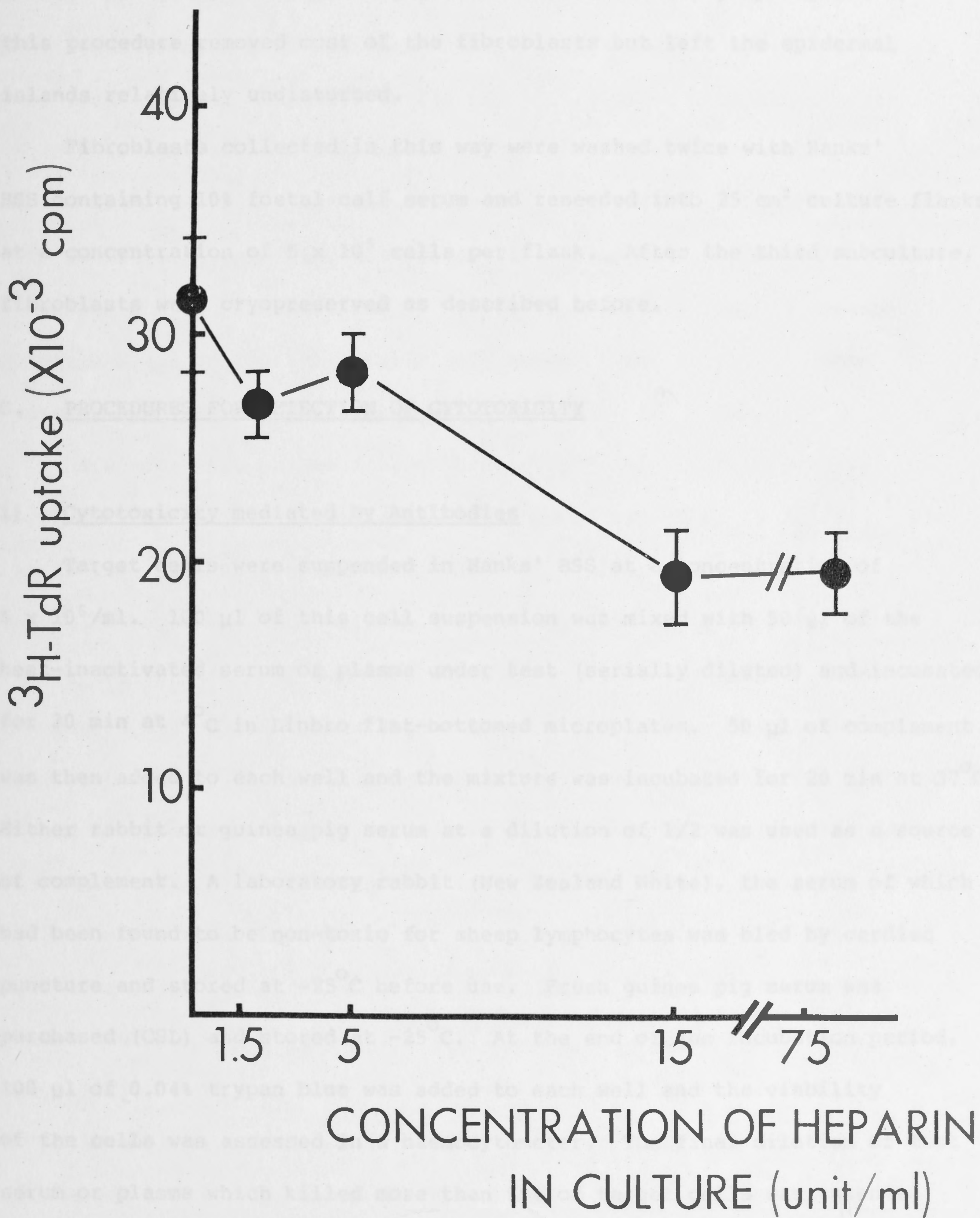
5) Establishment and Maintenance of Fibroblast Cultures from Foetal Skin

Foetal skin (3 x 1 cm) was obtained during the course of foetal thoracic duct cannulation. After the subcutaneous tissue was removed, a piece of skin was diced into fragments of approximately 1 x 1 x 1 mm using a sterile scalpel blade. Four to five fragments were placed in a 25 cm^2 tissue culture flask (#3013 Falcon Plastics) and overlaid with a ring of fresh sheep plasma which was allowed to clot at room temperature.

Plasma was harvested from fresh blood collected into sterile iced glass test tubes and immediately centrifuged at 800 G for 5 min. After the plasma overlaid on skin fragments had clotted, 10 ml of complete culture medium was added and the culture was incubated in an atmosphere of 10% CO_2, 7% O_2 and 83% N_2. The culture medium was changed every 7 days.

Fibroblasts and epithelial cells began to grow out from the skin fragments after 10 to 14 days of culture. When a cell monolayer covered

Figure C. Effect of serial dilutions of heparin on MLC. Sheep lymphocytes were cultured with allogeneic lymphocytes in the presence of various concentrations of heparin. Cultures were harvested on day 5 and results are expressed as mean cpm ± S.E. of triplicate cultures.



3H-TdR uptake (X10 3 cpm)
40
30
20
10
1.5
5
15
75
CONCENTRATION OF HEPARIN
IN CULTURE (unit/ml)

about 2/3 of the base of the flask, the culture medium was replaced with 10 ml of Hanks' BSS containing 0.25% trypsin (Difco) and 0.02% EDTA, prewarmed to 37°C. The adherent fibroblasts were dislodged within 3 to 4 min by this trypsinization, whilst epidermal cells showed a tendency to be dislodged later. Therefore, if properly timed, this procedure removed most of the fibroblasts but left the epidermal islands relatively undisturbed.

Fibroblasts collected in this way were washed twice with Hanks' BSS containing 10% foetal calf serum and reseeded into 25 cm^2 culture flasks at a concentration of 5×10^5 cells per flask. After the third subculture, fibroblasts were cryopreserved as described before.

G. PROCEDURES FOR DETECTION OF CYTOTOXICITY

1) Cytotoxicity mediated by Antibodies

Target cells were suspended in Hanks' BSS at a concentration of 5×10^6/ml. 100 µl of this cell suspension was mixed with 50 µl of the heat-inactivated serum or plasma under test (serially diluted) and incubated for 30 min at 4°C in Linbro flat-bottomed microplates. 50 µl of complement was then added to each well and the mixture was incubated for 20 min at 37°C. Either rabbit or guinea pig serum at a dilution of 1/2 was used as a source of complement. A laboratory rabbit (New Zealand White), the serum of which had been found to be non-toxic for sheep lymphocytes was bled by cardiac puncture and stored at -25°C before use. Fresh guinea pig serum was purchased (CSL) and stored at -25°C. At the end of the incubation period, 100 µl of 0.04% trypan blue was added to each well and the viability of the cells was assessed in a haemocytometer. The final dilution of test serum or plasma which killed more than 50% of target cells was taken as the antibody titre.

2) <u>Cell-mediated Cytolysis Assay</u>

i) ^{51}Cr release assay

Con A blasts, PHA blasts and foetal fibroblasts were used as target cells. Con A and PHA blasts were obtained by culturing lymphocytes for 3 days with 10 μg/ml of Con A (Sigma) and PHA-P (Difco) respectively. Foetal fibroblasts were obtained as described above. Target cells were incubated with from 200 to 250 μCi ^{51}Cr (Sodium chromate-(^{51}Cr)-injection, Australian Atomic Energy Commission, specific activity 157 mCi/mg) for 90 min at 37°C. After incubation the labelled cells were washed once with Hanks' BSS containing 10% foetal calf serum and subsequently incubated in complete culture medium for 20 min (this procedure reduced non-specific background radioactivity). Cells were washed twice more with Hanks' BSS containing 10% foetal calf serum and adjusted to 10^5 cells/ml.

Effector cells obtained from chronic lymphatic fistula were washed twice with Hanks' BSS and adjusted to a concentration of 10^7/ml. 0.1 ml of effector cell suspension was added to 0.1 ml of target cell suspension in a flat-bottomed Linbro microplate and cultured for 6 hr in an atmosphere of 10% CO_2 in air at 37°C. When effector cells were generated in MLC, Granberg's method (1980) was followed. 0.1 ml of supernatant was removed from each well of an MLC and the MLC microplates were shaken for 5 sec in a Vortex mixer after which 0.1 ml of the target cell suspension was added to each well. The microplates were then centrifuged at 150 G for 10 min prior to incubation at 37°C. At the end of this period, the microplates were centrifuged for 5 min at 150 G and the radioactivity in 0.1 ml of supernatant was measured in a gamma scintillation spectrophotometer (Packard, Model 578).

The maximum release of isotope from target cells was achieved by the incubation at 37°C for 6 hr of a mixture of 0.1 ml 2% Triton X100 and 0.1 ml of target cells. All the reactions were carried out in triplicate. The percentage cytotoxicity was determined as (experimental release -

spontaneous release/maximum release - spontaneous release) X 100.

ii) ^{3}H-Proline assay

Foetal fibroblasts were labelled with ^{3}H-Proline (NET-323 Proline, New England Nuclear) by the method of Bean, Kodera and Shiku (1976). 0.1 ml of labelled target cells were added to 0.1 ml of effector cells in Linbro microplates and cultured at 37°C for 20 hr in an atmosphere of 10% CO_2, 7% O_2 and 83% N_2. At the end of this period, 0.1 ml of culture supernatant was collected as described above and its radioactivity counted using a Packard Tri-Carb scintillation spectrophotometer. The scintillation fluid used was Aquasol (New England Nuclear).

H. STATISTICS

The significance of difference in means of various groups was assessed using Fisher's F test.

CHAPTER 1

THE REACTIVITY OF MATERNAL LYMPHOCYTES IN VITRO

A. INTRODUCTION

Although there have been numerous reports of the measurement of immunological reactivity during pregnancy, there is little consensus in the results (Beer and Billingham, 1976, Loke 1978, Stites et al. 1979, Rocklin, Kitzmiller and Kaye 1979). Using different experimental systems pregnancy has been reported as producing both increase and decrease in maternal reactivity against paternal tissues. It seems probable that inter-species variations in such factors as duration of pregnancy and the extent of placental separation of maternal and foetal circulations would account for some of the variations between reports, but convincing correlations are lacking. Many of the investigations of maternal immunological reactivity have been designed to test the proposition of Medawar (1953) that some form of immunological defect might be of importance in the ability of the mother to maintain a foetus which would otherwise elicit a response appropriate for a graft of foreign tissue. In most instances, maternal reactivity has been assessed by examination of the responsiveness in tissue culture of maternal lymphocytes and, consequently, it is often difficult to assess the relevance of those observations to the physiological situation in the whole animal.

Another common feature of the investigation of maternal immunological reactivity has been that examination on one or two occasions has often been extrapolated to infer that a similar level of responsiveness applies throughout pregnancy. Furthermore, investigations in non-inbred species have often been based on surveys of the response of a population rather than on comparison of responsiveness of the same individual throughout pregnancy. A difficulty encountered with the latter approach is that day-to-day variations are inherent in some of the in vitro assays.

The three aims of the experiments described in this chapter were to determine firstly whether any variations in the intrinsic reactivity of

lymphocytes collected before and during pregnancy from primiparous ewes could be detected, secondly the extent to which any modifications in reactivity were reproducible, and thirdly whether the *in vitro* reactivity of maternal lymphocytes was susceptible to modification by exposure to maternal serum or plasma. Cryopreservation of samples of lymphocytes collected at different stages of gestation so that comparative reactivity could be assessed in a single post-partum assay was an essential feature of these experiments. The use of cryopreservation techniques which permit the retention of lymphocyte function has permitted considerable reduction of the day-to-day variations otherwise experienced in repetitive testing of the proliferative responses of lymphocytes. The assays that were selected for sequential examination of maternal lymphocyte function in the present experiments included MLC to measure specific interparental immunological reactivity and non-specific blastogenesis assays in which the level of a general class of immunological reactivity could be followed.

B. RESULTS

1.1 THE OPTIMAL CONDITIONS FOR OVINE BLASTOGENESIS ASSAY AND MLC

a) Culture Medium and Supplementing Serum

To determine the combination of medium and serum which would support the maximal proliferation of cultivated lymphocytes, several combinations of Dulbecco's modification of Eagle's medium or of TC-199 medium (CSL) with supplement of foetal calf serum (FCS) or human serum were arbitrarily chosen and their ability to sustain the growth of sheep peripheral blood lymphocytes (PBL) was compared. As is shown in Figure 1.1, Dulbecco's medium supplemented with 10% FCS was superior to the other combinations and was accordingly used in subsequent experiments to test lymphocyte

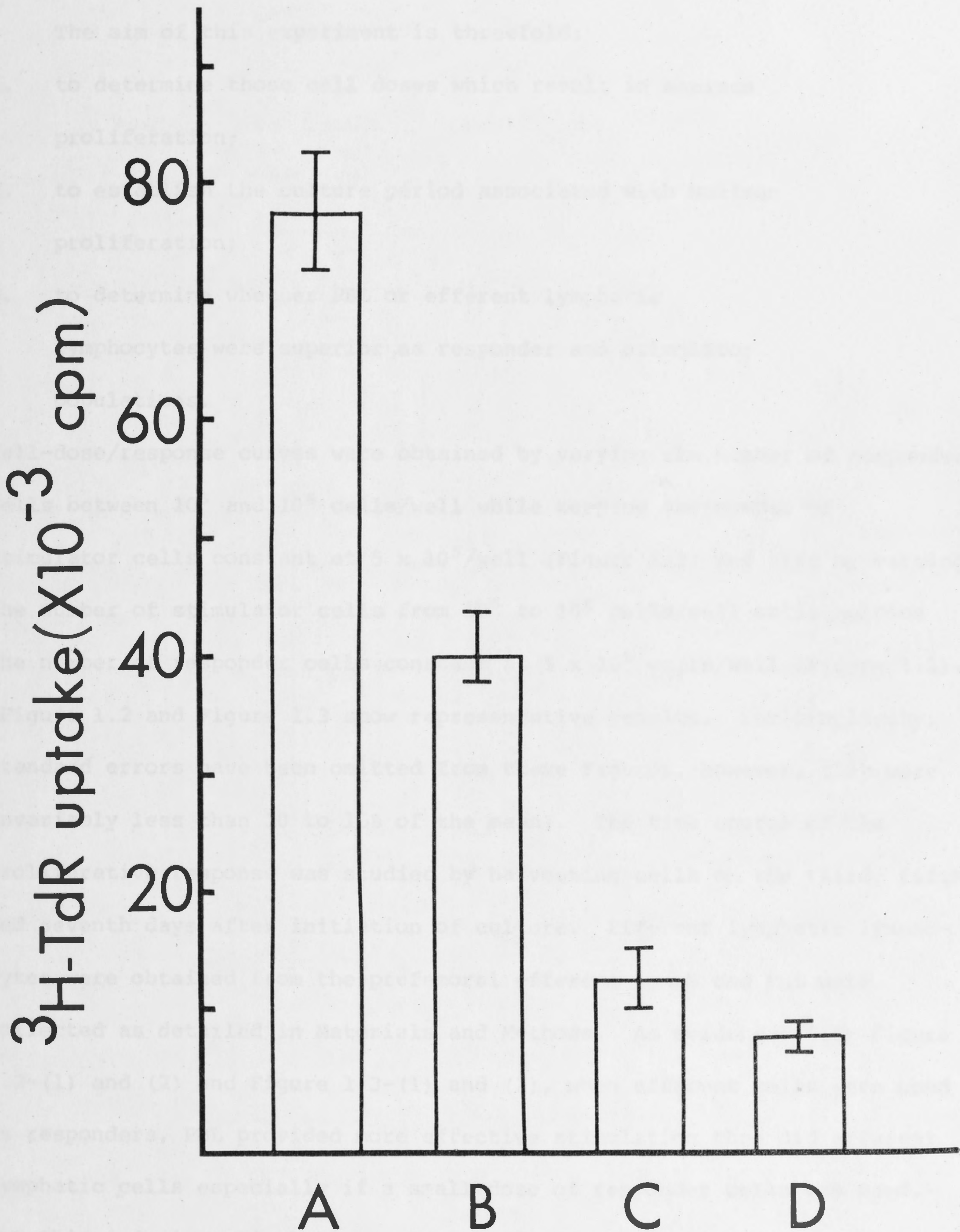
Figure 1.1 Comparison of efficacy of various combinations of culture medium and serum supplement. In each instance,a triplicate culture of 5 x 10^5 ovine PBL with 10 μg/ml Con A was established. Cultures were harvested after 3 days and results are expressed as mean cpm ± S.E.

A. Dulbecco's modified Eagle's medium (D-MEM) + 10% FCS.

B. D-MEM + 10% human serum.

C. TC-199 + 10% FCS.

D. TC-199 + 10% human serum.



3H-TdR uptake(X10-3 cpm)
80
60
40
20
A
B
C
D

proliferation. A single batch of FCS was used for each experiment.

b) The Cell-dose/Response Curves, Kinetics of Response and Comparative Efficiency of Different Cell Types as Responder and Stimulator Cells in MLC

The aim of this experiment is threefold;

1. to determine those cell doses which result in maximum proliferation;
2. to establish the culture period associated with maximum proliferation;
3. to determine whether PBL or efferent lymphatic lymphocytes were superior as responder and stimulator populations.

Cell-dose/response curves were obtained by varying the number of responder cells between 10^5 and 10^6 cells/well while keeping the number of stimulator cells constant at 5×10^5/well (Figure 1.2) and also by varying the number of stimulator cells from 10^5 to 10^6 cells/well while holding the number of responder cells constant at 5×10^5 cells/well (Figure 1.3). (Figure 1.2 and Figure 1.3 show representative results. For simplicity, standard errors have been omitted from these Figures, however, they were invariably less than 10 to 15% of the mean). The time course of the proliferative response was studied by harvesting cells on the third, fifth and seventh days after initiation of culture. Efferent lymphatic lymphocytes were obtained from the prefemoral efferent lymph and PBL were collected as detailed in Materials and Methods. As evidenced from Figure 1.2-(1) and (2) and Figure 1.3-(1) and (2), when efferent cells were used as responders, PBL provided more effective stimulation than did efferent lymphatic cells especially if a small dose of responder cells was used. But this inferior stimulating capacity of efferent cells was not apparent if PBL were used as responders (Figure 1.2-(3) and (4) and Figure 1.3-(3) and (4). That is, efferent cells could effectively stimulate allogeneic

Figure 1.2 Dose-response characteristics and comparative responsiveness of efferent lymphatic cells and PBL. Each group of 3 columns represents mean cpm of triplicate cultures of 3, 5 and 7 days duration respectively. The number of responder cells was:

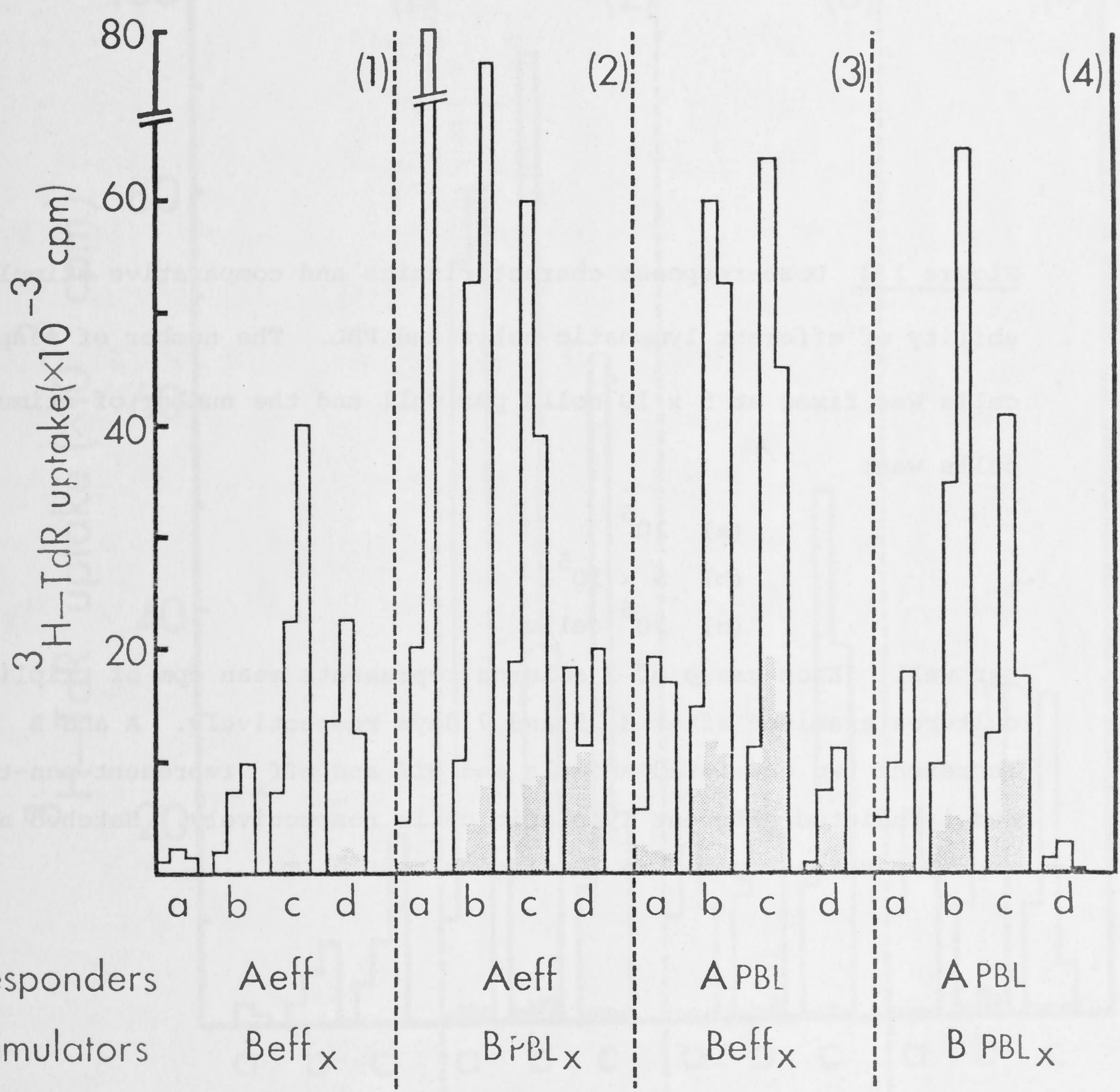
(a) 10^5

(b) 2.5×10^5

(c) 5×10^5

(d) 10^6

cells per well. The number of stimulator cells was constant at 5×10^5 cells per well. A and B represent two unrelated animals and eff and eff_x represent non-treated and irradiated efferent lymphatic cells respectively. The hatched area represents mean cpm of an autologous control.

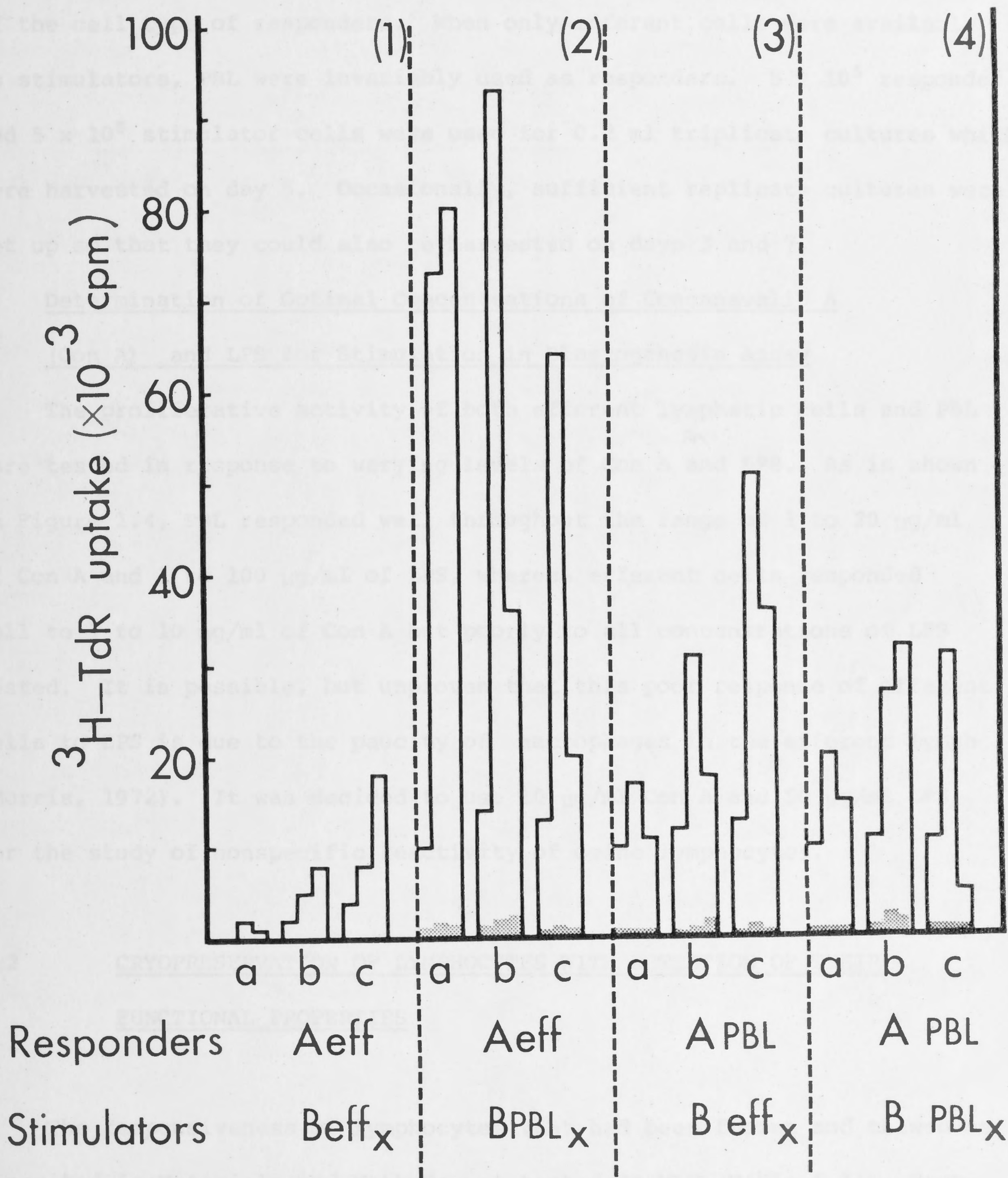


80
60
40
20
3H–TdR uptake(×10 −3 cpm)
(1)
(2)
(3)
(4)
a b c d a b c d a b c d a b c d
Responders
Stimulators
Aeff
Beff x
Aeff
B PBL x
A PBL
Beff x
A PBL
B PBL x

Figure 1.3 Dose-response characteristics and comparative stimulating ability of efferent lymphatic cells and PBL. The number of responder cells was fixed at 5×10^5 cells per well and the number of stimulator cells was:

(a) 10^5,
(b) 5×10^5,
(c) 10^6 cells

per well. Each group of 3 columns represents mean cpm of triplicate cultures examined after 3, 5 and 7 days respectively. A and B represent two unrelated animals and eff and eff_x represent non-treated and irradiated efferent lymphatic cells respectively. Hatched area represents mean cpm of an autologous control.



100
80
60
40
20
^{3}H–TdR uptake (×10^3 cpm)
(1)
(2)
(3)
(4)
a b c
a b c
a b c
a b c
Responders
Aeff
Aeff
A PBL
A PBL
Stimulators
Beff$_x$
BPBL$_x$
B eff$_x$
B PBL$_x$

lymphocytes provided these were PBL. Peak reactivity was observed at either day 5 or day 7 of culture irrespective of the dose of cells used. Peak responses were never observed before day 5 unless sensitized cells were used as responders as discussed later. On the basis of these results, it was decided to use PBL as stimulators in general regardless of the cell type of responders. When only efferent cells were available as stimulators, PBL were invariably used as responders. 5×10^5 responder and 5×10^5 stimulator cells were used for 0.2 ml triplicate cultures which were harvested on day 5. Occasionally, sufficient replicate cultures were set up so that they could also be harvested on days 3 and 7.

c) Determination of Optimal Concentrations of Concanavalin A (Con A) and LPS for Stimulation in Blastogenesis Assay

The proliferative activity of both efferent lymphatic cells and PBL were tested in response to varying levels of Con A and LPS. As is shown in Figure 1.4, PBL responded well throughout the range of 1 to 20 μg/ml of Con A and 1 to 100 μg/ml of LPS, whereas efferent cells responded well to 1 to 10 μg/ml of Con A but poorly to all concentrations of LPS tested. It is possible, but unproven that this poor response of efferent cells to LPS is due to the paucity of macrophages in the efferent lymph (Morris, 1972). It was decided to use 10 μg/ml Con A and 50 μg/ml LPS for the study of nonspecific reactivity of ovine lymphocytes.

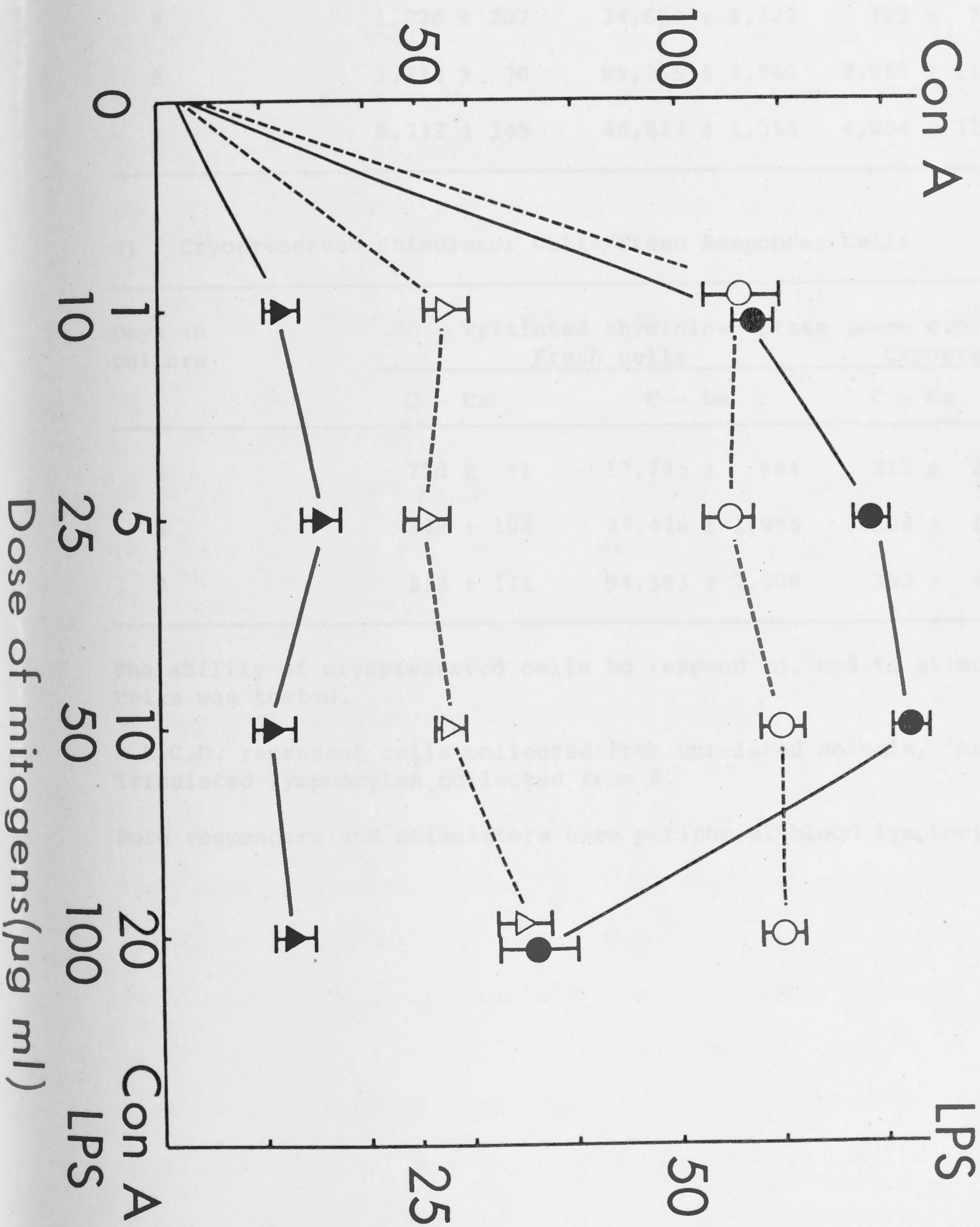
1.2 CRYOPRESERVATION OF LYMPHOCYTES WITH RETENTION OF THEIR FUNCTIONAL PROPERTIES

The responsiveness of lymphocytes that had been frozen and thawed as described in Materials and Methods was tested in MLC (Table 1.1). Cryopreserved cells seemed to possess a marginally decreased ability to respond to and stimulate allogeneic cells but the kinetics of the response resembled that of fresh cells. To compensate for this marginal loss of

Figure 1.4 Dose-response characteristics of sheep lymphocytes in response to Con A and LPS after 3 days of culture. Results are expressed as mean cpm ± S.E.

● Response of efferent lymphatic cells to Con A,
○ response of PBL to Con A,
▲ response of efferent lymphatic cells to LPS,
△ response of PBL to LPS.

Left ordinate indicates scale of responsiveness to Con A and right ordinate responsiveness to LPS.



3H-TdR uptake (X10-3 cpm)
Con A
50
100
LPS
25
50
Dose of mitogens (µg ml)
Con A
1
5
10
20
LPS
10
25
50
100

Table 1.1 Comparative reactivity of fresh cells and cryopreserved cells.

1) Cryopreserved Responder Cells/Fresh Stimulator Cells

Days in culture	Tritiated thymidine uptake (mean c.p.m. ± S.E.)			
	Fresh cells		Cryopreserved cells	
	A - Ax	A - Bx	A - Ax	A - Bx
3	1,220 ± 207	34,664 ± 3,122	795 ± 74	30,953 ± 430
5	1,215 ± 70	99,365 ± 3,540	2,860 ± 110	85,779 ± 1,931
7	6,732 ± 145	46,817 ± 1,351	4,004 ± 314	48,257 ± 3,482

2) Cryopreserved Stimulator Cells/Fresh Responder Cells

Days in culture	Tritiated thymidine uptake (mean c.p.m. ± S.E.)			
	Fresh cells		Cryopreserved cells	
	C - Cx	C - Dx	C - Cx	C - Dx
3	720 ± 41	13,745 ± 964	211 ± 25	11,428 ± 905
5	349 ± 104	27,446 ± 1,956	124 ± 10	20,641 ± 2,239
7	213 ± 111	94,563 ± 7,200	153 ± 44	92,005 ± 3,875

The ability of cryopreserved cells to respond to, and to stimulate, allogeneic cells was tested.

A.B.C.D. represent cells collected from unrelated animals, 'Ax' indicates irradiated lymphocytes collected from A.

Both responders and stimulators were peripheral blood lymphocytes.

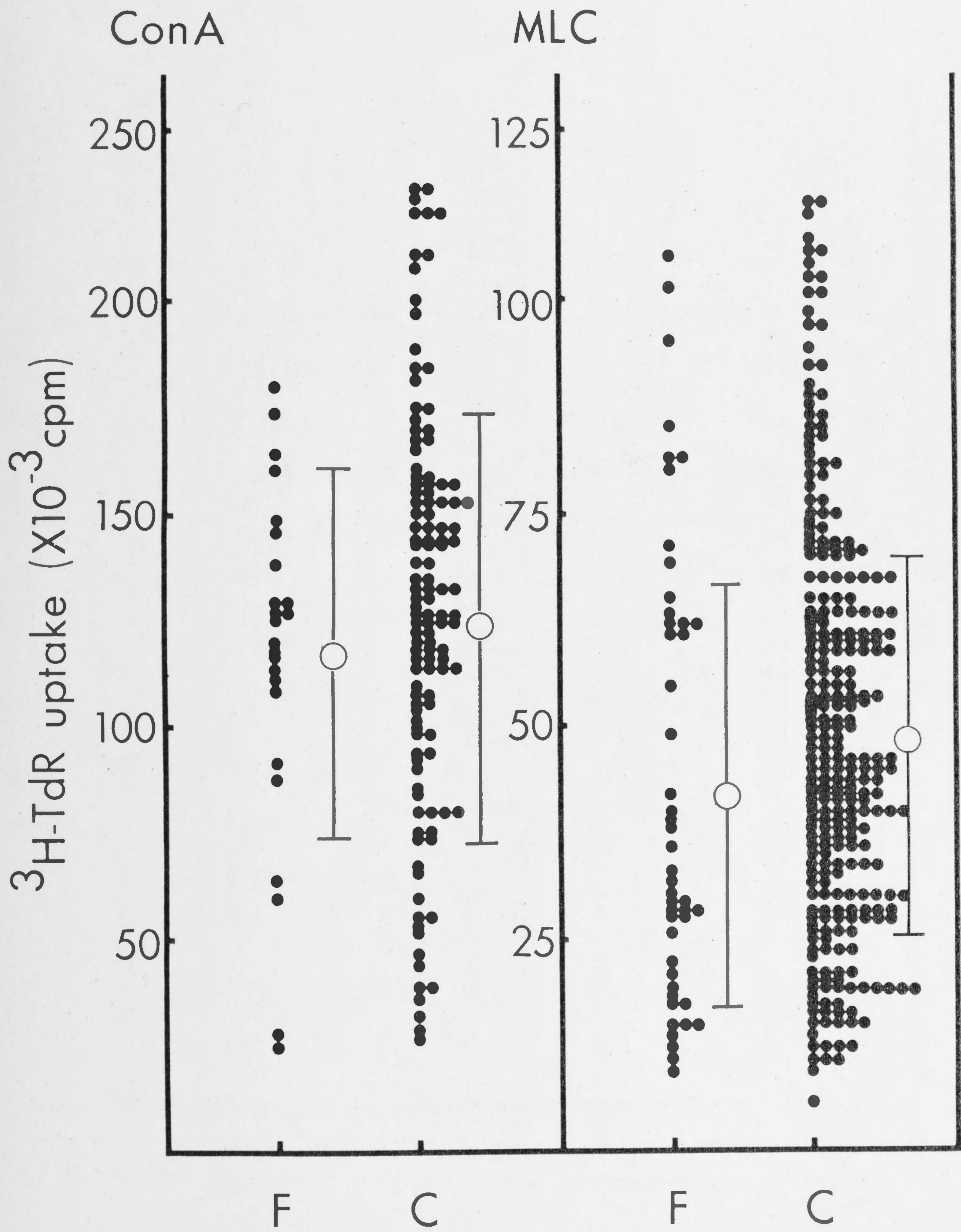
capacity, 6×10^5 cryopreserved cells were substituted for 5×10^5 both as responders and stimulators and good MLC responses with unaltered kinetics were obtained. Figure 1.5 summarizes the results of testing 24 fresh and 122 frozen samples of either PBL or efferent cells in Con A blastogenesis assay and 51 fresh and 259 frozen samples of either PBL or efferent cells in MLC. On the whole, frozen cells responded to Con A and to allogeneic cells as well as fresh cells and no statistically significant difference was observed ($p > 0.01$). Peak reactivity in MLC was observed between day 5 and day 7 in every case in which the time course of the response was followed.

1.3 THE DEMONSTRATION OF 'FACTOR OF IMMUNIZATION' IN MLC

As an objective of subsequent MLC assays was to determine whether cells from pregnant donors had been subject to primary stimulus before examination, it was necessary to examine whether primary and secondary responses in MLC could be readily distinguished.

The effect of immunization on the responsiveness of lymphocytes in MLC was studied using cells sensitized *in vitro* and *in vivo*. To test the effects of sensitization, normal popliteal efferent cells collected from animal A were cultured with irradiated allogeneic PBL collected from animals B and C (Figure 1.6). The peak response was observed on day 6 in this primary MLC after which those cells that had been cultured with irradiated PBL from animal B were washed, adjusted in number and exposed again on day 7 to irradiated PBL from either of animals B or C. By this time their peak response had diminished. These primed cells started to proliferate earlier in response to the second encounter with the immunogen and attained a peak response on day 3 of the secondary MLC, whereas the response of B-primed cells to cells from the unrelated animal C followed a normal time course.

Figure 1.5 Individual responses of fresh and cryopreserved cells in Con A blastogenesis assay and MLC. Results are expressed as individual responses with mean cpm ± S.D. F and C on the abscissa represent fresh and cryopreserved cells respectively. Left column of the graph represents Con A and right column MLC response. Both efferent lymphatic cells and PBL were used as responder cells.


ConA
MLC
^{3}H-TdR uptake (X10^{-3}cpm)
250
200
150
100
50
125
100
75
50
25
F
C
F
C

<u>Figure 1.6</u> Kinetics of primary and secondary MLC. Popliteal efferent lymphatic cells collected from animal A were cultured with irradiated PBL collected form animal B or animal C. On day 7 of the primary MLC, cells which had been cultured with irradiated PBL from animal B were washed, adjusted in number and re-stimulated with irradiated PBL from either animal B or animal C. Results are expressed as mean cpm of triplicate cultures.

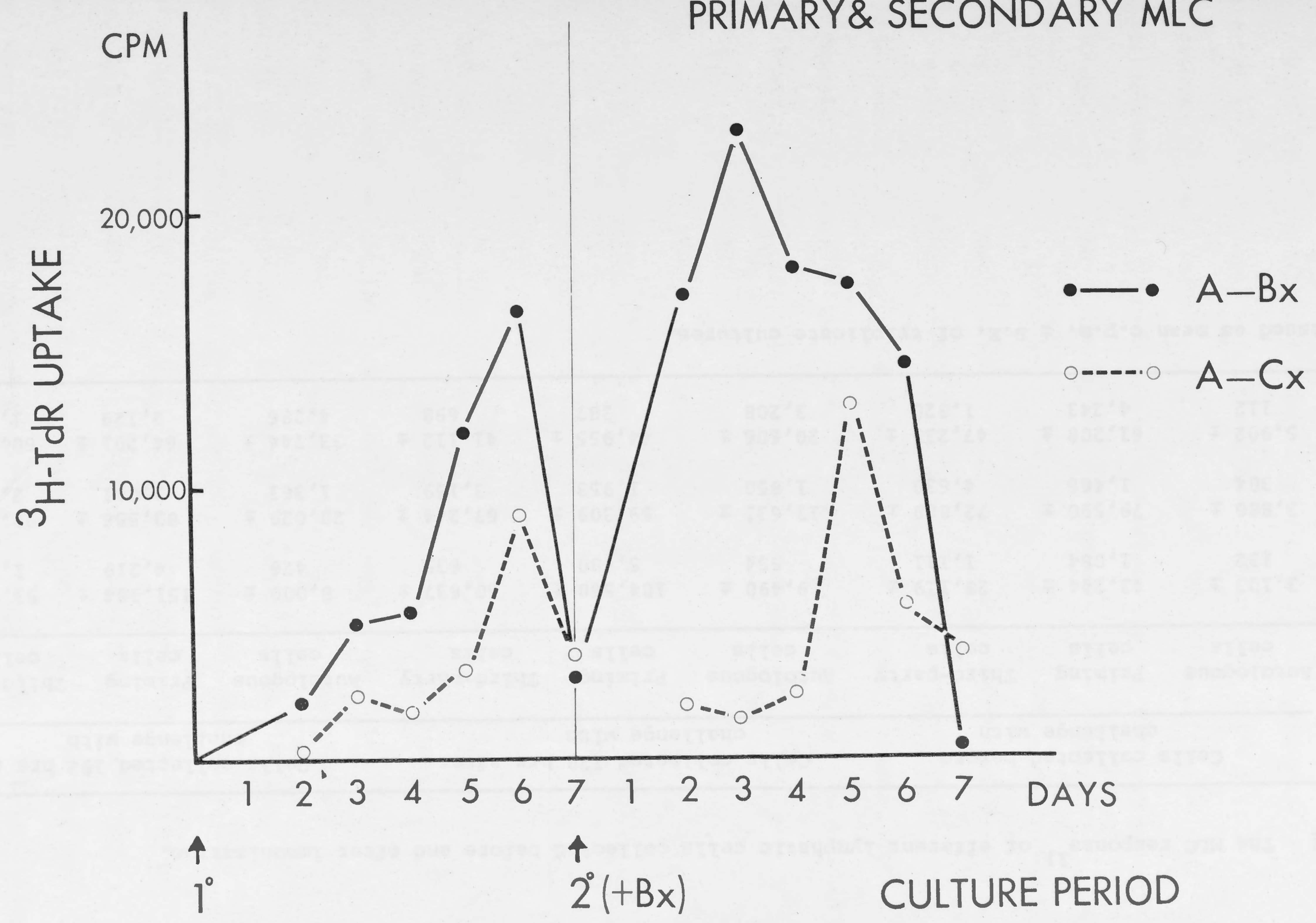

PRIMARY& SECONDARY MLC
CPM
20,000
10,000
3H-TdR UPTAKE
A—Bx
A—Cx
1 2 3 4 5 6 7 1 2 3 4 5 6 7
DAYS
1°
2°(+Bx)
CULTURE PERIOD

Table 1.2 The MLC response[1] of efferent lymphatic cells collected before and after immunization.

Days in culture	Cells collected before challenge with			Cells collected 120 hrs after challenge with			Cells collected 192 hrs after challenge with		
	Autologous cells	Priming cells	Third-party cells	Autologous cells	Priming cells	Third-party cells	Autologous cells	Priming cells	Third-party cells
3	3,103 ± 132	43,244 ± 1,084	28,119 ± 1,111	9,490 ± 554	104,568 ± 5,480	50,637 ± 635	8,000 ± 426	151,184 ± 4,219	53,972 ± 1,752
5	3,880 ± 304	79,590 ± 1,466	72,869 ± 4,630	17,631 ± 1,850	59,309 ± 1,953	67,244 ± 3,159	20,029 ± 1,363	83,556 ± 3,061	92,943 ± 2,248
7	5,902 ± 112	61,208 ± 4,243	47,235 ± 1,320	20,606 ± 3,208	44,955 ± 387	41,112 ± 698	33,744 ± 4,286	64,201 ± 2,129	60,119 ± 1,210

1) Expressed as mean c.p.m. ± S.E. of triplicate cultures.

In the case of in vivo sensitization, normal ewes with popliteal lymphatic fistulae were inoculated subcutaneously in the lower leg with 6 x 10^7 allogeneic lymphocytes. The efferent cells subsequently collected from the fistula were cultured with stimulator cells obtained from either the donor animal or an unrelated animal. Sensitization with allogeneic cells in vivo both increased the proliferation of lymphocytes in response to a second challenge with the primary cells in vitro and also accelerated their response to the extent that its peak was attained on day 3 of culture (Table 1.2). In contrast, the reactivity of the primed cells against unrelated cells reached its peak on day 5.

In summary, both in vitro and in vivo primed lymphocytes commenced cell division two days earlier than did unprimed cells and also manifested significantly higher levels of proliferation when challenged with the priming cells in vitro.

1.4 MLC REACTIVITY OF PBL FROM PRIMIPAROUS EWES AGAINST FOETAL, PATERNAL AND UNRELATED CELLS

a) MLC Reactivity of Jugular PBL

Seventeen virgin Merino and cross-bred ewes were mated with either Merino or Merino-Border Leicester crossbred rams. Jugular venous blood samples were obtained from each ewe before mating, on from 3 to 8 occasions during pregnancy, and in the post partum period. PBL were separated from each of these specimens, cryopreserved and subsequently tested in MLC assay against PBL from both paternal and unrelated third party donors. PBL collected at each time from 5 of the ewes were also tested against paternal efferent lymphocytes, while cells from 7 of the ewes were assayed against foetal thoracic duct lymphocytes. All collections of PBL from 8 of the ewes were also tested against efferent lymphatic lymphocytes from unrelated third party donors. Because it was not

technically feasible at the time when these experiments were conducted to separate sufficient PBL from foetal blood, foetal PBL were not used as stimulators. Representative results are shown in Figure 1.7. Examination of the reactivity against paternal PBL of the cells in 79 collections from primiparous ewes revealed significant variation from the pre-pregnant levels in only two instances; namely a reduction of responsiveness of PBL collected from one ewe on the 140th and 150th days of pregnancy (Figure 1.7-d). Similarly, apart from the two instances cited above, the MLC reactivity of maternal cells against third party PBL did not vary significantly throughout pregnancy. The peak response of maternal cells generally occurred after 5 days of culture, apart from occasional cases in which peak reactivity was delayed until the seventh day. However, third-day peak responses as observed with presensitized cells were never encountered.

Similar assays were performed with the substitution, as stimulators, of lymphocytes collected from either an efferent lymphatic of the ram or the foetal thoracic duct (Figure 1.8). As noted in Materials and Methods, efferent cells were inferior to PBL as stimulators. However, in contrast with the consistent results obtained throughout pregnancy when paternal PBL were used as stimulators, variations in responsiveness during pregnancy were frequent if either paternal efferent cells or foetal thoracic duct lymphocytes were substituted as stimulators. Foetal thoracic duct lymphocytes were used on 7, and paternal lymphatic efferent lymphocytes on 5 occasions as stimulators of maternal PBL. Cells from 6 of the 10 donor ewes examined expressed significantly augmented MLC reactivity as pregnancy progressed, when tested against these stimulator cells. The magnitude of the increases (in cpm) ranged from 2 to 10 fold (Figure 1.8-a, b, c). Similarly increased responses were also observed if efferent lymphatic lymphocytes from unrelated third party donors were used as stimulators (Figure 1.8-a,b).

Figure 1.7 Examples of mixed lymphocyte reactivity (against paternal and third-party PBL) of maternal PBL collected throughout pregnancy. In each instance, triplicate culturesof 6×10^5 maternal cells were established with 5×10^5 irradiated autologous cells or cells from a paternal or third-party donor. Cultures were harvested after 5 days and results are expressed as means alone, standard errors having been omitted for clarity. The arrow of each horizontal axis denotes the time of parturition.

□ irradiated autologous cells,

● irradiated paternal cells,

○ irradiated third-party cells.

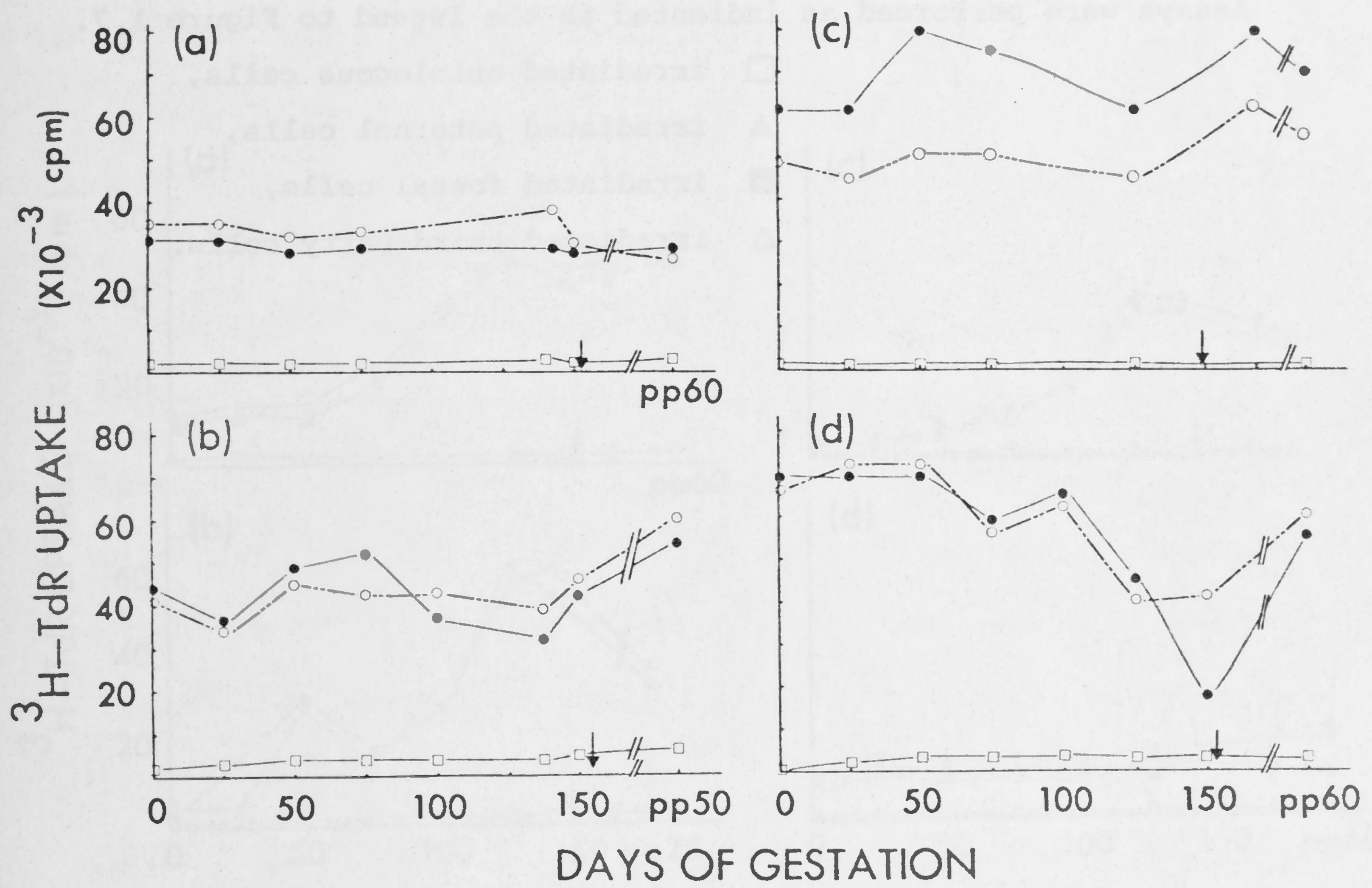

80
60
40
20
(a)
pp60
(b)
(c)
(d)
^{3}H–TdR UPTAKE (X10^{-3} cpm)
0
50
100
150
pp50
pp60
DAYS OF GESTATION

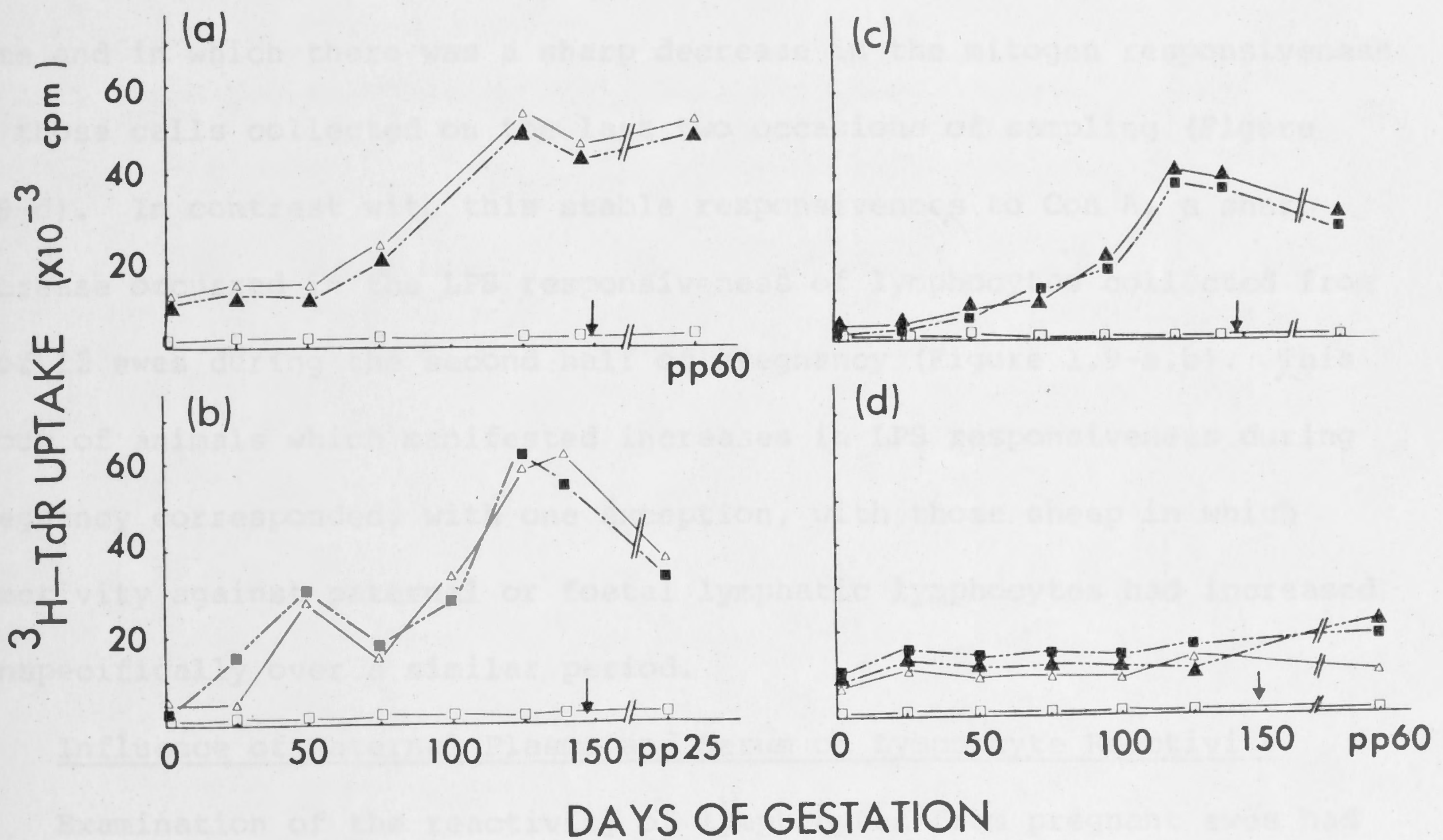
Figure 1.8 Examples of mixed lymphocyte reactivity against efferent lymphatic lymphocytes of maternal PBL collected throughout pregnancy. Assays were performed as indicated in the legend to Figure 1.7.

□ irradiated autologous cells,

▲ irradiated paternal cells,

■ irradiated foetal cells,

△ irradiated third-party cells.


(a)
(b)
(c)
(d)
60
40
20
3H–TdR UPTAKE (X10-3 cpm)
pp60
0
50
100
150
pp25
DAYS OF GESTATION

The increase in the responsiveness of maternal PBL to stimulation by paternal efferent or foetal thoracic duct lymphocytes during pregnancy generally brought these responses to levels almost comparable to those attained by similar cells in response to paternal PBL.

b) Reactivity of Jugular PBL to Nonspecific Mitogens

The responsiveness to Con A and LPS of maternal PBL collected throughout pregnancy was examined (Figure 1.9). Responsiveness to Con A of cells from 14 out of 15 ewes remained remarkably uniform throughout pregnancy. The only exception was that animal in which a marked reduction in MLC reactivity against paternal PBL had been observed at a similar time and in which there was a sharp decrease in the mitogen responsiveness of those cells collected on the last two occasions of sampling (Figure 1.9-d). In contrast with this stable responsiveness to Con A, a sharp increase occurred in the LPS responsiveness of lymphocytes collected from 7 of 12 ewes during the second half of pregnancy (Figure 1.9-a,b). This group of animals which manifested increases in LPS responsiveness during pregnancy corresponded, with one exception, with those sheep in which reactivity against paternal or foetal lymphatic lymphocytes had increased nonspecifically over a similar period.

c) Influence of Maternal Plasma and Serum on Lymphocyte Reactivity

Examination of the reactivity of lymphocytes from pregnant ewes had not, to this point, indicated that any significant variation from the functional state existing before pregnancy had occurred. However, it remained possible that successive collections of cells which had been found to express similar, unaltered reactivity in vitro might have responded differently in the donor. For instance, it has been suggested that the apparent non-reactivity of some types of tolerant animals and tumor-bearing humans may reflect the presence of humoral blocking factors sufficient to mask a state of cellular immunity (Hellström, Hellstrom and Brawn 1969, Voisin, Kinsky and Due 1972). By extrapolation it could be suggested

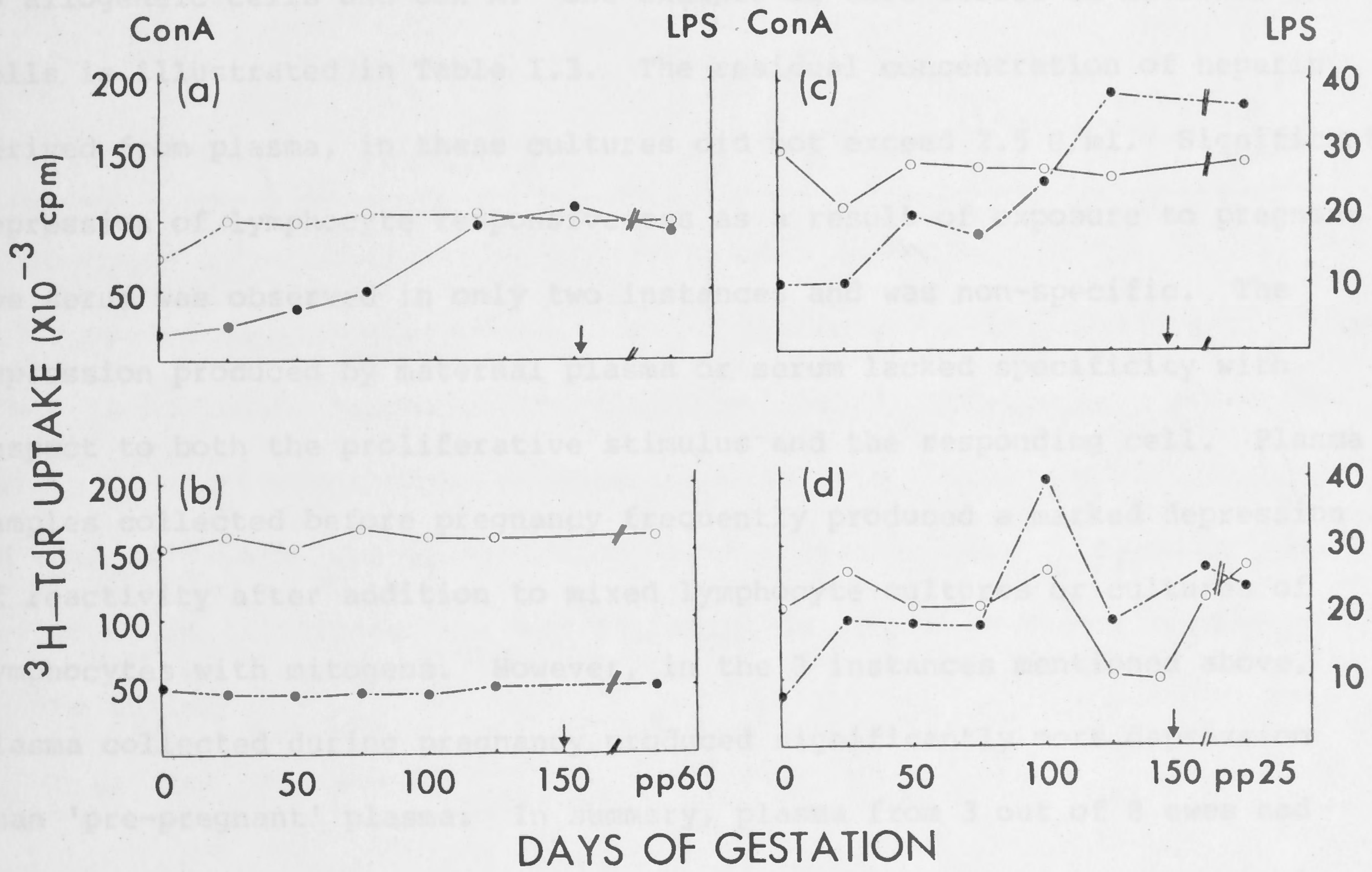
Figure 1.9 Examples of responsiveness to mitogens of maternal PBL collected throughout pregnancy. Assays were performed in triplicate to

○ Con A,

● LPS.

Left ordinate indicates scale of responsiveness to Con A and right ordinate that to LPS.

that lymphocytes from a pregnant ewe, although fully reactive *in vitro*, might be subject to inhibitory humoral influences whilst in the environment of the pregnant donor. To explore this possibility, the effect on *in vitro* lymphocyte reactivity of plasma collected from 8 ewes, and of serum collected from 14 ewes on a number of occasions during pregnancy, was tested (3 animals were common to both groups). Plasma collected from 3 ewes during the last third of pregnancy produced significant depression of the response of both maternal and third-party lymphocytes to allogeneic cells and Con A. One example of this effect on maternal cells is illustrated in Table 1.1. The residual concentration of heparin, derived from plasma, in these cultures did not exceed 1.5 U/ml. Significant depression of lymphocyte responsiveness as a result of exposure to pregnant ewe serum was observed in only two instances and was non-specific. The depression produced by maternal plasma or serum lacked specificity with respect to both the proliferative stimulus and the responding cell. Plasma samples collected before pregnancy frequently produced a marked depression of reactivity after addition to mixed lymphocyte cultures or cultures of lymphocytes with mitogens. However, in the 3 instances mentioned above, plasma collected during pregnancy produced significantly more depression than 'pre-pregnant' plasma. In summary, plasma from 3 out of 8 ewes had suppressive activity whilst serum from 2 out of 14 animals was suppressive. Suppressive activity was not detected in any of the specimens from the 3 ewes which had provided both plasma and serum. The infrequency with which serum or plasma from pregnant donors modified the *in vitro* reactivity of lymphocytes suggests that reactivity as expressed in culture is likely to resemble that *in vivo*.



d) Relative Reactivity of Maternal Lymphocytes returning from the Uterine and General Circulation.

It remained possible that lymphocytes returning from the uterus might display specific or nonspecific alterations in reactivity that would

that lymphocytes from a pregnant ewe, although fully reactive in vitro, might be subject to inhibitory humoral influences whilst in the environment of the pregnant donor. To explore this possibility, the effect on in vitro lymphocyte reactivity of plasma collected from 8 ewes, and of serum collected from 14 ewes on a number of occasions during pregnancy, was tested (3 animals were common to both groups). Plasma collected from 3 ewes during the last third of pregnancy produced significant depression of the response of both maternal and third-party lymphocytes to allogeneic cells and Con A. One example of this effect on maternal cells is illustrated in Table 1.3. The residual concentration of heparin, derived from plasma, in these cultures did not exceed 2.5 U/ml. Significant depression of lymphocyte responsiveness as a result of exposure to pregnant ewe serum was observed in only two instances and was non-specific. The depression produced by maternal plasma or serum lacked specificity with respect to both the proliferative stimulus and the responding cell. Plasma samples collected before pregnancy frequently produced a marked depression of reactivity after addition to mixed lymphocyte cultures or cultures of lymphocytes with mitogens. However, in the 3 instances mentioned above, plasma collected during pregnancy produced significantly more depression than 'pre-pregnant' plasma. In summary, plasma from 3 out of 8 ewes had suppressive activity whilst serum from 2 out of 14 animals was suppressive. Suppressive activity was not detected in any of the specimens from the 3 ewes which had provided both plasma and serum. The infrequency with which serum or plasma from pregnant donors modified the in vitro reactivity of lymphocytes suggests that reactivity as expressed in culture is likely to resemble that in vivo.

d) Relative Reactivity of Maternal Lymphocytes returning from the Uterine and General Circulation

It remained possible that lymphocytes returning from the uterus might display specific or nonspecific alterations in reactivity that would

Table 1.3 Effect of plasma from pregnant ewes on lymphocyte reactivity.

Plasma collection (of pregnancy day)	Tritiated thymidine uptake (mean c.p.m. ± S.E.) in response to		
	(a) Autologous cells	(b) Paternal cells	(c) Con A
Before pregnancy	411 ± 133	28,141 ± 3,356	29,490 ± 3,464
87	121 ± 24	22,752 ± 2,475	8,964 ± 608
100	71 ± 15	26,245 ± 2,877	7,980 ± 573
129	121 ± 44	9,296 ± 1,291	7,580 ± 524
145	111 ± 16	12,800 ± 271	10,644 ± 354
10 (post-partum)	297 ± 43	14,214 ± 2,190	n.d.[1)]

Lymphocytes obtained from efferent lymph of a ewe before pregnancy were incorporated as responder cells in mixed lymphocyte cultures and exposed to Con A in media incorporating 10% plasma collected from the same ewe before, during or after pregnancy. Stimulator cells were autologous efferent cells in (a) and paternal peripheral blood lymphocytes in (b). All assays were performed in triplicate, (a) and (b) being harvested on the fifth day and (c) on the third day.

1) n.d. not determined.

remain undetectable in populations of lymphocytes collected from other sites in the circulatory system because of dilution by unmodified cells. Populations of lymphocytes returning from the uterus could, hypothetically, be either enriched with, or selectively depleted of, specifically immunized cells if selective addition or retention of cells specifically reactive against foetal determinants had occurred in the course of exposure of maternal cells during passage through the placenta.

To test the possibility that lymphocytes returned from the uterus with modified reactivity, blood samples were collected from the uterine vein of 6 ewes, ranging in duration of pregnancy from 40 to 92 days (preliminary experiments entailing cannulation of uterine lymphatics afferent to the regional nodes were discontinued because lymph from these vessels yielded insufficient cells for performance of the required assays). Mononuclear cells separated from blood samples simultaneously collected from uterine and jugular veins were incorporated into mixed lymphocyte cultures against paternal or third-party PBL or foetal thoracic duct cells as stimulators (Table 1.4). Uterine cells were also incubated with Con A and LPS (Table 1.5) to provide a 'baseline' indication of nonspecific responsiveness that would readily permit detection of any change in specific reactivity. In almost all instances, lymphocytes from uterine and jugular veins behaved indistinguishably in both types of assay, there being no indication of any significant difference in potential reactivity.

e) Effect of Plasma from the Uterine Vein on Maternal Lymphocyte Reactivity

The preceding experiments gave no indication that lymphocytes returning from the gravid uterus differed in MLC reactivity from cells in the general circulation. To exclude the possibility that the normal responsiveness of uterine vein cells in culture did not accurately reflect their capacity in the donor, the influence of plasma derived from the same uterine vein blood samples on their reactivity was examined. As summarized in

Table 1.4 Comparative reactivity of maternal jugular and uterine vein lymphocytes in mixed lymphocyte culture.

Pregnant ewe	Stage of pregnancy (days)	Tritiated thymidine uptake (mean c.p.m. ± S.E. ($x10^{-3}$)) against (1) paternal (P) or foetal (F) cells of uterine PBL	jugular PBL	(2) third party P.B.L. of uterine PBL	jugular PBL
48M	40	41.5 ± 2.2	43.9 ± 2.7 (P)	40.3 ± 0.4	45.3 ± 1.0
29M	62	56.1 ± 1.5	40.7 ± 5.3 (F)	42.5 ± 4.5	45.6 ± 3.0
35M	65	39.3 ± 3.2	41.8 ± 1.3 (F)	53.8 ± 2.5	57.3 ± 1.6
38M	73	40.7 ± 0.9	64.2 ± 2.1 (P)	33.1 ± 0.4	49.3 ± 1.7
40M	75	36.2 ± 3.1	30.3 ± 2.9 (P)	27.5 ± 1.4	22.0 ± 1.9
39M	92	53.1 ± 1.2	61.9 ± 0.3 (P)	52.8 ± 1.9	53.8 ± 3.8

Lymphocytes harvested from uterine or jugular vein blood at the indicated stage of pregnancy were employed as responder cells. Stimulator cells were peripheral blood lymphocytes in paternal and third party situations, and thoracic duct lymphocytes in the case of the foetus. All assays were undertaken in triplicate and harvested on the fifth day.

PBL: peripheral blood lymphocytes.

Table 1.5 Comparative reactivity of maternal jugular and uterine vein lymphocytes to Concanavalin A and lipopolysaccharide.

Pregnant ewe	Stage of pregnancy (days)	Tritiated thymidine uptake (mean c.p.m. ± S.E. ($x10^{-3}$)) against Concanavalin A of uterine PBL	against Concanavalin A of jugular PBL	against Lipopolysaccharide of uterine PBL	against Lipopolysaccharide of jugular PBL
48M	40	75.5 ± 1.3	78.0 ± 3.9	8.6 ± 0.8	5.9 ± 0.3
29M	62	167.5 ± 7.1	217.7 ± 3.6	20.8 ± 0.2	30.8 ± 1.8
30M	63	122.0 ± 4.5	128.1 ± 7.5	69.6 ± 0.6	27.4 ± 1.5
35M	65	118.1 ± 5.0	233.1 ± 4.0	41.2 ± 1.2	33.3 ± 0.3
38M	73	218.2 ± 4.6	183.2 ± 3.9	2.3 ± 0.2	6.0 ± 0.5
40M	75	129.7 ± 3.6	124.7 ± 1.5	10.0 ± 0.8	6.9 ± 0.4
39M	92	215.8 ± 3.2	218.8 ± 1.1	23.7 ± 0.2	27.9 ± 0.4

Lymphocytes were harvested from uterine or jugular vein blood at the indicated stage of pregnancy. All assays were undertaken in triplicate and harvested on the third day.

PBL peripheral blood lymphocytes.

Table 1.6, uterine vein plasma was indistinguishable from jugular vein plasma in its influence when added to mixed lymphocyte cultures.

f) Stimulatory Capacity of Maternal Lymphocytes during Pregnancy

The responsiveness of maternal lymphocytes has been described in sections (a), (b) and (d). Lymphocytes carry histocompatibility antigens and are able to stimulate other lymphocytes of genetically different origin (Bach and Hirschhorn 1964). Any changes in stimulatory capacity of maternal cells during pregnancy could be relevant given the fact that the ovine foetal lymphoid system is competent to recognize alloantigens, at least in the later half of pregnancy (Silverstein, Prendergast and Kraner 1964). Maternal PBL were collected from 7 ewes before conception, on 4 to 6 occasions during pregnancy, and in the post partum period and cryopreserved. The stimulatory activity of these cells for paternal and foetal and third party lymphocytes as responder cells was tested in MLC. As shown in Figure 1.10, maternal stimulating capacity was well maintained throughout pregnancy and there was no indication of maternal 'inertia' in terms of presenting alloantigens to lymphocytes of unrelated paternal or foetal animals.

DISCUSSION

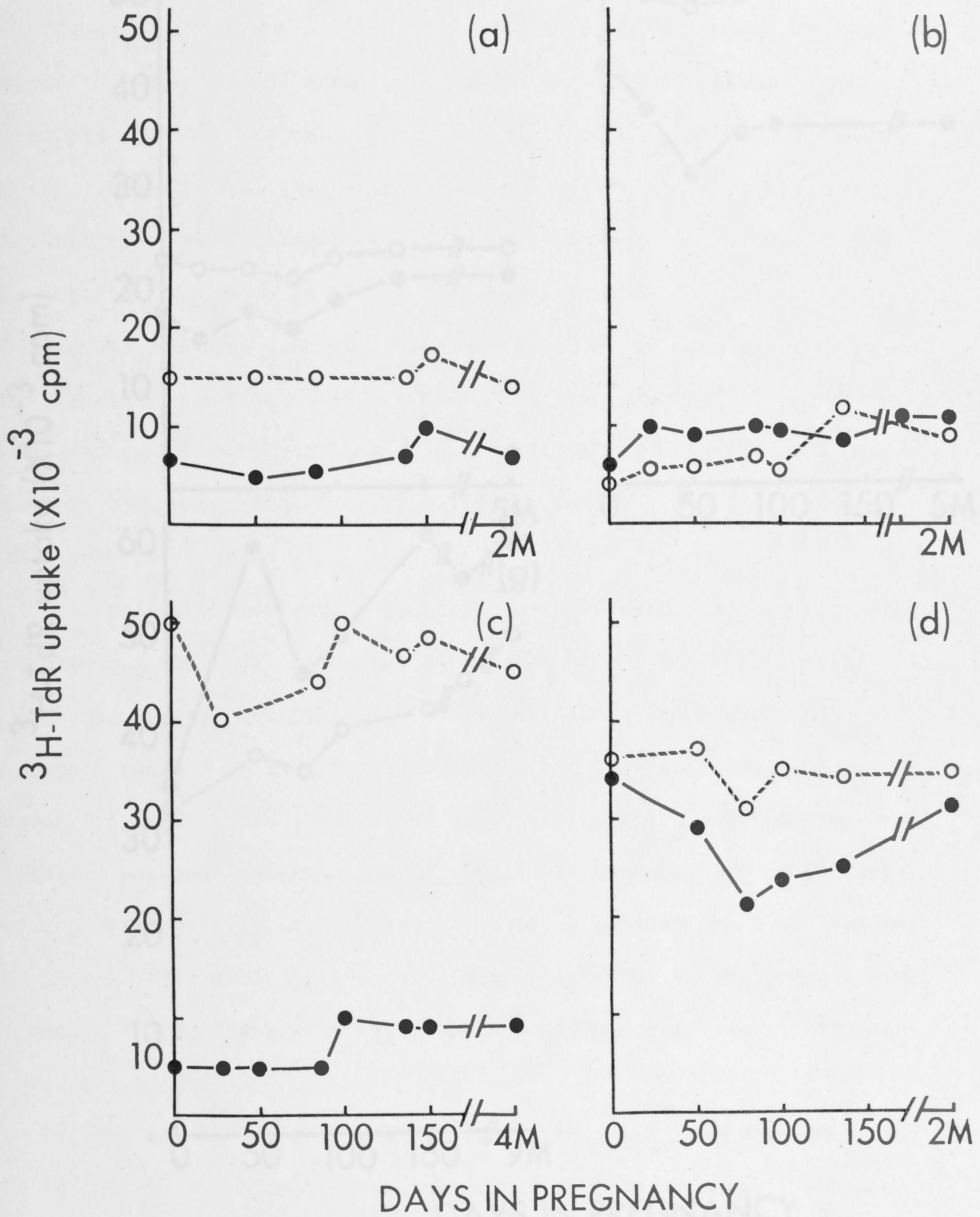
The reactivity of maternal lymphocytes against paternal and third-party PBL and against Con A did not vary significantly during pregnancy. However, in some ewes, there was a marked increase in the responsiveness of maternal lymphocytes towards foetal thoracic duct, paternal and third-party efferent lymphatic lymphocytes and LPS during pregnancy. There was no indication that the reactivity of the lymphocyte population returning from the gravid uterus differed from that of lymphocytes from other areas of the maternal circulation. Whilst sera collected at different stages of pregnancy from a group of ewes showed little capacity to interfere with

Table 1.6 Influence of plasma from maternal jugular or uterine veins on the response of maternal lymphocytes in mixed lymphocyte culture.

Pregnant plasma donor	Stage of pregnancy (days)	Tritiated thymidine uptake (mean c.p.m. ± S.E. ($x10^{-3}$)) against Paternal PBL in presence of		Third-party PBL in presence of	
		Uterine plasma	Jugular plasma	Uterine plasma	Jugular plasma
29M	62	4.1 ± 0.3	4.0 ± 0.5	4.7 ± 0.1	5.1 ± 0.4
30M	63	19.1 ± 1.9	19.3 ± 0.6	15.0 ± 1.0	19.0 ± 1.1
35M	65	29.9 ± 1.1	29.7 ± 2.1	24.3 ± 1.6	1[illegible].2 ± 0.5
38M	73	23.3 ± 0.1	23.4 ± 1.4	21.7 ± 0.2	22.7 ± 1.3
40M	75	33.2 ± 0.2	28.5 ± 1.9	32.6 ± 1.5	18.4 ± 1.0

Lymphocytes obtained from the efferent lymph of each plasma donor ewe before pregnancy were incorporated as responder cells in mixed lymphocyte cultures the media of which incorporated 10% plasma collected from a jugular or uterine vein at the indicated state of pregnancy. All cultures were undertaken in triplicate and harvested on the fifth day.

Figure 1.10 Stimulatory activity of maternal PBL collected throughout pregnancy. Each group (a) to (g) represents the data from an individual ewe. PBL were collected on the days specified and their stimulatory activity in MLC was tested. Responder cells were paternal efferent lymphatic cells (●) in (a), (b), (c), (d) and (g), foetal thoracic duct lymphocytes (●) in (e) and (f), and efferent lymphatic cells from an unrelated animal (○) in (a) - (g). Results are expressed as mean cpm of triplicate cultures harvested on day 5.



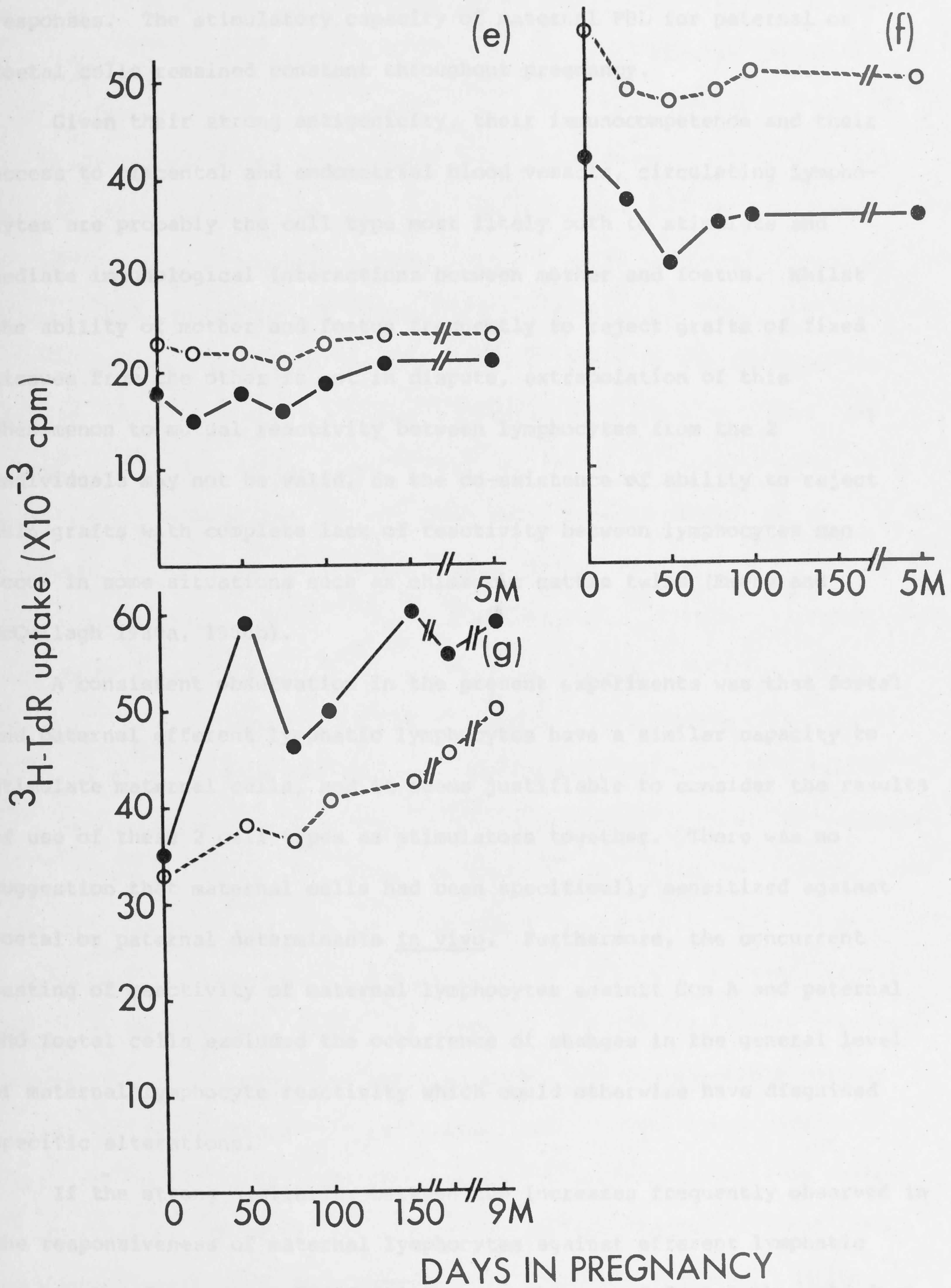
(a)
(b)
(c)
(d)
50
40
30
20
10
^{3}H-TdR uptake (X10^{-3} cpm)
0
50
100
150
2M
4M
DAYS IN PREGNANCY

the in vitro reactivity of maternal lymphocytes, some specimens of plasma collected in the later stage of pregnancy did reduce these responses. The stimulatory capacity of maternal PBL for paternal or foetal [illegible] remained constant throughout pregnancy.

Given their strong antigenicity, their immunocompetence and their access to [illegible]cental and endometrial blood vessels, circulating lymphocytes are probably the cell type most likely both to [illegible] and mediate [illegible]logical interactions between mother and foetus. Whilst the ability of mother and foetus [illegible] to reject grafts of fixed tissues [illegible] the other is not in dispute, extrapolation of this phenomenon to mutual reactivity between lymphocytes from the 2 individuals may not be valid, as the co-existence of ability to reject [illegible]grafts with complete lack of reactivity between lymphocytes can occur in some situations such as chimaeric cattle twins [illegible] McCullagh [illegible]).

A consistent observation in the present experiments was that foetal and maternal efferent [illegible]matic lymphocytes have a similar capacity to stimulate maternal cells, and it seems justifiable to consider the results of use of these 2 cell types as stimulators together. There was no suggestion that maternal cells had been specifically sensitised against foetal or paternal determinants in vivo. Furthermore, the concurrent testing of [illegible]activity of maternal lymphocytes against Con A and paternal and foetal cells excluded the occurrence of changes in the general level of maternal [illegible]phocyte reactivity which could otherwise have disguised specific alterations.

If the [illegible] increases frequently observed in the responsiveness of maternal lymphocytes against efferent lymphatic lymphocytes from paternal and third-party donors and foetal thoracic duct cells (all poor stimulator populations), and also against LPS, as pregnancy progressed, is to be adequately explained, it is necessary to



the in vitro reactivity of maternal lymphocytes, some specimens of plasma collected in the later stage of pregnancy did reduce these responses. The stimulatory capacity of maternal PBL for paternal or foetal cells remained constant throughout pregnancy.

Given their strong antigenicity, their immunocompetence and their access to placental and endometrial blood vessels, circulating lymphocytes are probably the cell type most likely both to stimulate and mediate immunological interactions between mother and foetus. Whilst the ability of mother and foetus frequently to reject grafts of fixed tissues from the other is not in dispute, extrapolation of this phenomenon to mutual reactivity between lymphocytes from the 2 individuals may not be valid, as the co-existence of ability to reject skin grafts with complete lack of reactivity between lymphocytes can occur in some situations such as chimaeric cattle twins (Emery and McCullagh 1980a, 1980b).

A consistent observation in the present experiments was that foetal and paternal efferent lymphatic lymphocytes have a similar capacity to stimulate maternal cells, and it seems justifiable to consider the results of use of these 2 cell types as stimulators together. There was no suggestion that maternal cells had been specifically sensitized against foetal or paternal determinants in vivo. Furthermore, the concurrent testing of reactivity of maternal lymphocytes against Con A and paternal and foetal cells excluded the occurrence of changes in the general level of maternal lymphocyte reactivity which could otherwise have disguised specific alterations.

If the strong similarity between the increases frequently observed in the responsiveness of maternal lymphocytes against efferent lymphatic lymphocytes from paternal and third-party donors and foetal thoracic duct cells (all poor stimulator populations), and also against LPS, as pregnancy progressed, is to be adequately explained, it is necessary to

understand the poor stimulatory capacity of efferent lymphocyte populations for cells from non-pregnant donors. Two general, and not necessarily exclusive, explanations could be that there is some universal deficiency in the properties of the cell population or that the content of a subpopulation of cells with strong stimulatory capacity is reduced. The present experiments do not provide any assistance in distinguishing between these alternatives. It could be envisaged that pregnancy exerts its effect on the responsiveness of maternal cells to efferent cells by circumventing the mechanism responsible for their deficiency as stimulators.

In extrapolation from the present experiments to the intact pregnant ewe, it is possible that, although maternal lymphocytes responded normally to cultured foetal cells, their ability to recognize foetal determinants might be impaired in the intact animal by influences which were not reproducible *in vitro*. The suggestion, obtained from some experiments, that maternal plasma could depress lymphocyte reactivity may have resulted from an activity of heparin similar to that reported by Currie (1967). However, the much more marked effect resulting from the use of plasma obtained from the same ewe during pregnancy compared with the activity of pre-pregnant plasma does not support this interpretation. Experiments in which the response of maternal peripheral lymph nodes to challenge with foetal lymphocytes was examined (described in Chapter 4) have not given any indication that responsiveness of the ewe is impaired. A more difficult objection to overcome is that the nature of any modification to maternal lymphocytes during pregnancy could be such as to be detectable only by assays that demonstrate previous sensitization of the lymphocytes under test. If there are major histocompatibility differences between mother and foetus, it may be difficult to demonstrate a 'factor of immunization' by conventional tests, but this does not guarantee that modification of lymphocyte responsiveness has not occurred. Given the earlier demonstration that preliminary sensitization between unrelated sheep could be readily

detected by its effect on the kinetics of the MLC, it is unlikely that any sensitization induced by pregnancy would have been overlooked.

It was shown that the stimulatory capacity of maternal PBL for foetal or paternal cells was well maintained throughout pregnancy, implying that maternal cells would provide adequate stimulation of the foetal immune system following their introduction into the foetus. The ability of the foetus to develop an immune response against its mother is discussed later.

The present results appear to exclude the occurrence of any specific modification of the reactivity of those maternal lymphocytes passing through the circulation to the uterus and placental tissue. If cells passing through this region had been specifically sensitized, or if a local humoral factor had impaired lymphocyte reactivity at this site alone, it is likely that examination of the reactivity of cells collected from the uterine veins would have revealed some alteration.

CHAPTER 2

THE IN VIVO REACTIVITY OF THE EWE TOWARDS PATERNAL AND FOETAL CELLS AND TISSUES

A. INTRODUCTION

Maternal lymphocytes were shown in Chapter 1 to be fully capable of responding to paternal and foetal cells in vitro. Although no indication of interference with this reactivity by maternal plasma or serum was obtained, the possibility remained that recognition of foetal determinants by maternal lymphocytes in vivo might be impaired by influences which, whilst operative in the animal, could not be demonstrated in vitro.

This chapter reports the results of an investigation of alloreactivity in primiparous ewes, particularly in relation to the foetus and ram. It was considered that the most appropriate way in which to assess maternal reactivity in vivo was the method described by Hall and Morris (1962). An efferent lymphatic of the popliteal lymph node was cannulated so that the immune response in the node could be continuously monitored under physiological conditions for long periods of time and examination of both morphological and functional attributes of lymphocytes migrating in response to stimulation became possible. The lymph node was stimulated by injecting either foetal or paternal lymphocytes subcutaneously or by placing foetal skin grafts on the lower leg. This approach has been used previously to provide a more informative record of the homograft reaction than that available from macroscopic observation of the graft (Hall 1967).

B. RESULTS

2.1 ABSENCE OF SPONTANEOUSLY OCCURRING ANTIBODIES CYTOTOXIC FOR PATERNAL LEUCOCYTES FROM THE PLASMA OF PRIMIGRAVID EWES

As indicated in the Introduction, cytotoxic antileucocyte antibodies have been detected in the serum of parous sheep (Ford and Elves

1974). It appeared likely from the results presented that these antibodies had been produced as a response to parturition rather than to pregnancy itself, but as none of the data presented was derived from ewes during a first pregnancy, this point could not be resolved.

To clarify whether production of anti-paternal antibodies was a feature of the first pregnancy, 104 plasma specimens collected from 18 ewes before, during and after pregnancy were tested against paternal PBL for the presence of cytotoxic antibody as described in Materials and Methods (Table 2.1). In no instance could cytotoxic activity be demonstrated at the starting dilution (1:2). Confirmation of the adequacy of the protocol for demonstrating cytotoxic antibody was obtained by examination of lymph collected from popliteal lymph nodes challenged with foetal or paternal cells in Section 2.2 (b).

2.2 STIMULATION OF MATERNAL POPLITEAL LYMPH NODE WITH FOETAL OR PATERNAL LYMPHOCYTES

a) Cellular Responses

Ewes were inoculated subcutaneously in the lower leg with foetal or paternal cells to test the capacity of the local lymphoid tissue to respond to foetal or paternal determinants.

Lymph was collected from a popliteal fistula and the number and type of cells were determined as described in Materials and Methods. Ten pregnant ewes at varying stages (from 93 to 127 days) of pregnancy and 2 non-pregnant ewes were tested. The results are summarized in Table 2.2 and the kinetics of the response of representative animals are shown in Figure 2.1 for non-pregnant and in Figure 2.2 for pregnant animals.

Measurement of the reactivity of pregnant ewes to foetal lymphocytes was limited by the availability of stimulating cells. In non-pregnant animals, there was initially a considerable increase in the output of small

Table 2.1 Cytotoxicity of plasma collected from primigravid ewes.

Animal	Days of collection in pregnancy	Antibody titre	Animal	Days of collection in pregnancy	Antibody titre
3M	0	0	27M	0	0
	87	0		21	0
	100	0		44	0
	129	0		76	0
	145	0		105	0
	+10[1]	0		119	0
7M	0	0		+2M	0
	123	0	28M	0	0
	+2	0		21	0
8M	0	0		76	0
	128	0		99	0
	140	0		114	0
	+7M[2]	0		142	0
5M	92	0		+2M	0
	120	0	31M	0	0
	141	0		25	0
	+6M	0		50	0
6M	0	0		75	0
	50	0		100	0
	87	0		125	0
	100	0	34M	0	0
	125	0		25	0
	+6M	0		50	0
26M	0	0		75	0
	22	0		100	0
	75	0		136	0
	104	0		148	0
	134	0		+1M	0
	+3M	0			
36M	0	0	80M	29	0
	25	0		50	0
	50	0		75	0
	75	0		100	0
	100	0		+1M	0
	135	0	82M	0	0
	145	0		25	0

Table 2.1 (Cont.)

Animal	Days of collection in pregnancy	Antibody titre	Animal	Days of collection in pregnancy	Antibody titre
37M	0	0		100	0
	50	0		+5d	0
	87	0	84M	0	0
	100	0		75	0
	125	0		100	0
	+2M	0		125	0
39M	0	0		150	0
	25	0		+1M	0
	75	0	86M	0	0
	100	0		42	0
	140	0		71	0
	150	0		100	0
40M	0	0		125	0
	25	0		148	0
	50	0		+2M	0
	75	0	Popliteal lymph of 31M collected 7 days after challenge with paternal PBL		>1 : 16
	100	0			
	125	0	Popliteal lymph of 37M collected 7 days after challenge with paternal PBL		>1 : 16

Paternal PBL (5×10^5/0.1 ml) were incubated with 50 µl heat-inactivated test plasma for 30 min at 4°C. 50 µl fresh guinea pig serum at a dilution of ½ was added. The incubation was continued for 20 min at 37°C. Cytotoxicity was determined by trypan blue exclusion.

1) 10 days postpartum.

2) 7 months postpartum.

Table 2.2 Maternal popliteal lymph node response towards foetal or paternal lymphocytes.

Ewe	Stage of pregnancy (days)	Source of cells for challenge	Dose ($x10^{-8}$)	Max. total cell output ($x10^{-7}$/hr)	Max. blast cell output ($x10^{-5}$/hr)	Cytotoxic antibody[1)] (Log_2 titre)
3M	Not pregnant	Allo. eff.[3)]	2.0	22.3	16.5	>4
IM-R	Not pregnant	Allo. eff	2.5	7.5	21.4	n.d.[2)]
IM-L	Not pregnant	Allo. PBL	2.0	7.5	27.9	n.d.
58M	93	Pat. PBL[4)]	0.6	22.5	48.2	>4
60M	96	Pat. PBL	0.6	7.8	5.7	>4
57M	97	Pat. PBL	0.6	10.9	5.8	>4
29M	112	Foetal TDL[5)]	1.0	22.7	12.7	>4
23M	116	Foetal TDL	0.6	12.5	11.7	>4
50M	118	Foetal TDL	0.6	13.8	15.2	>4
35M	122	Foetal TDL	1.5	16.4	30.1	0
38M	124	Pat. eff.[6)]	1.0	20.2	36.5	>4
40M	124	Foetal TDL	1.5	13.6	24.6	0
24M	127	Foetal TDL	1.0	13.0	23.9	>4

1) Cytotoxic antibody activity was measured against the priming cells. In 29M, 23M and 50M foetal fibroblasts obtained from each foetus were also used as target cells and the same titres were obtained.

2) Not determined.

3) Allogeneic lymphatic efferent cells.

4) Paternal PBL.

5) Foetal thoracic duct lymphocytes.

6) Paternal lymphatic efferent cells.

Figure 2.1 Popliteal lymph node response of non-pregnant ewes to allogeneic cells. Animals were injected with:

(a) 2×10^8 efferent lymphatic cells,
(b) 2×10^8 efferent lymphatic cells,
(c) 2.5×10^8 PBL

from unrelated donors. Total cell output from the regional node is plotted as a single line and blast cell response as a hatched area.

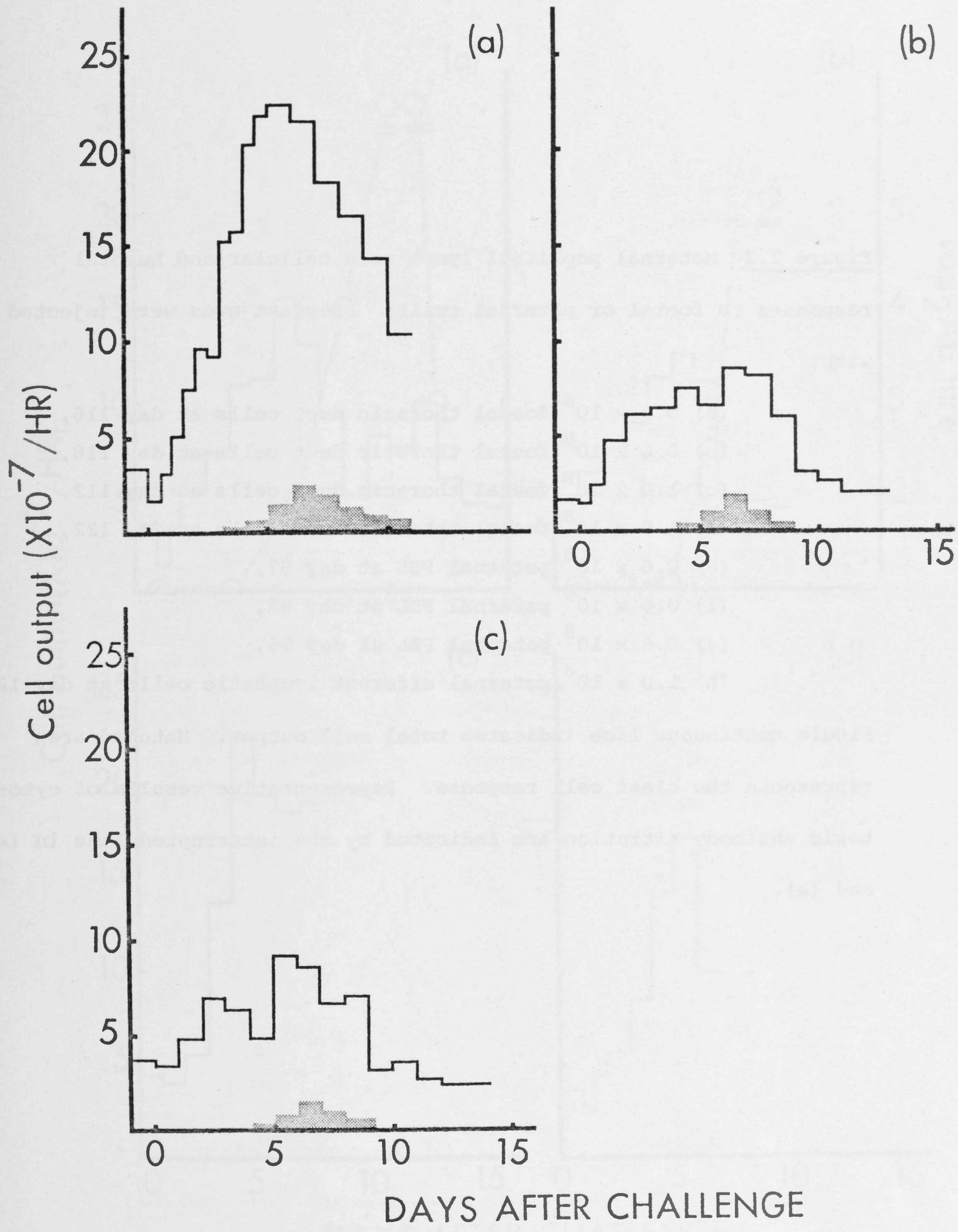
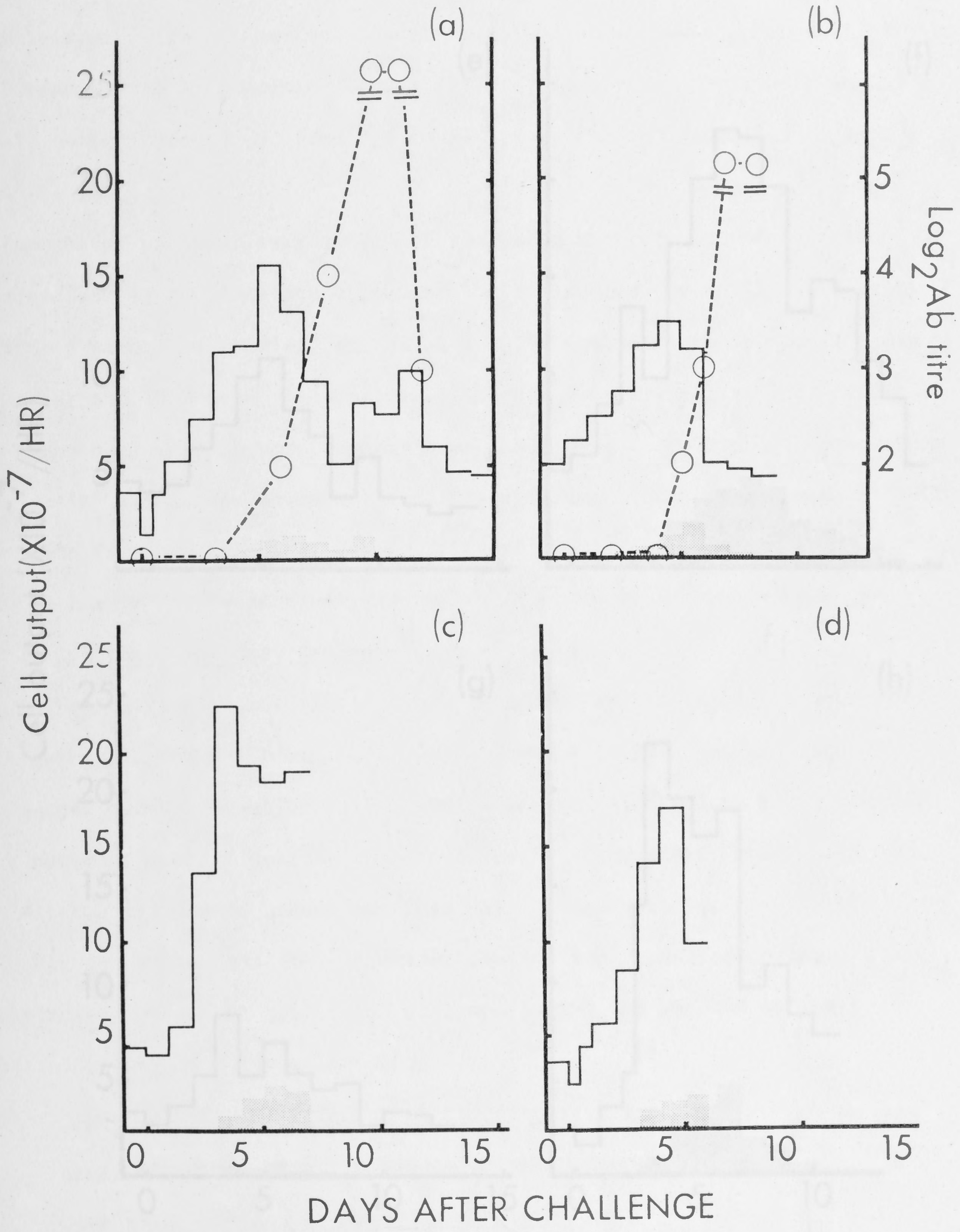
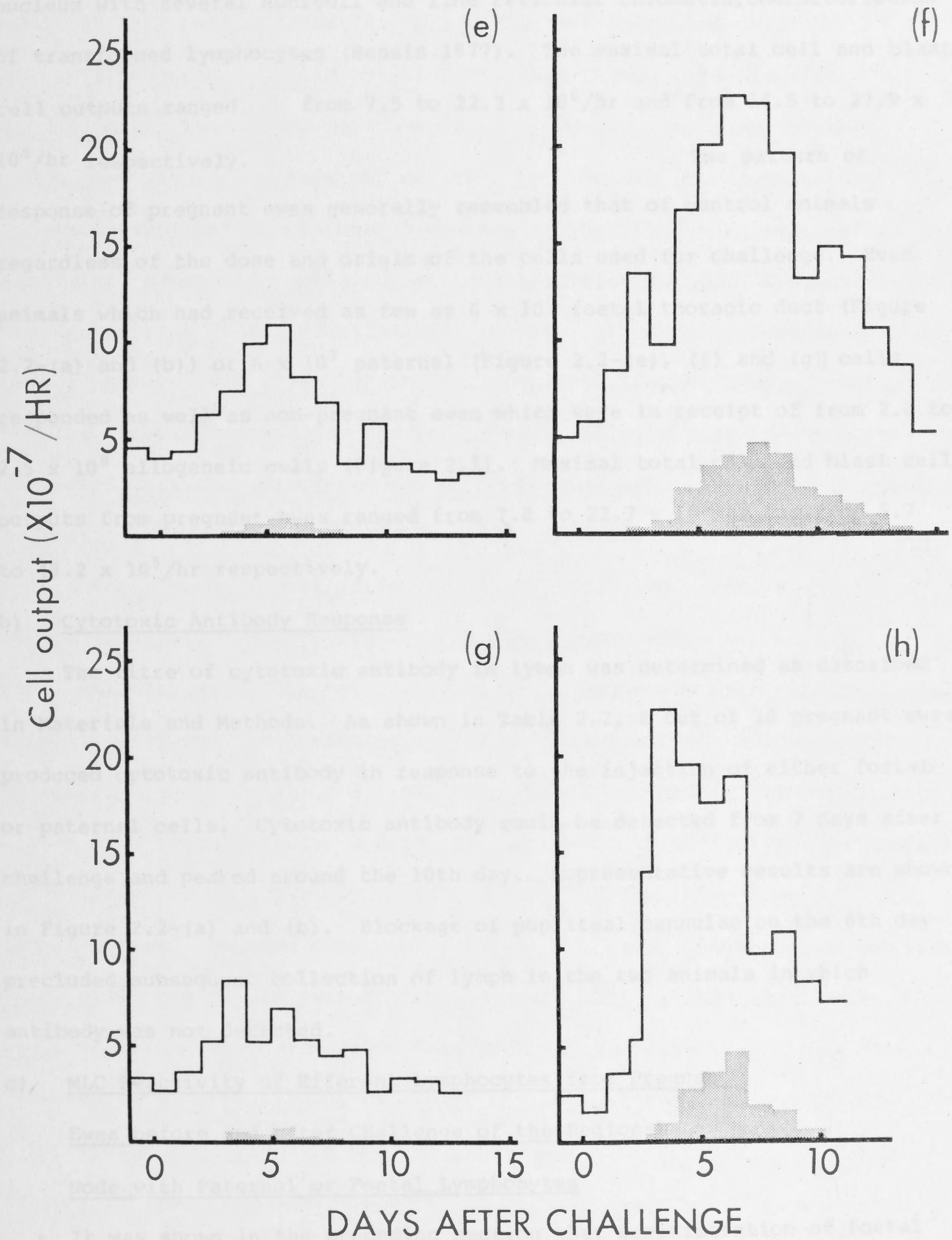

(a)
(b)
(c)
Cell output (X10⁻⁷/HR)
25
20
15
10
5
0
5
10
15
DAYS AFTER CHALLENGE

Figure 2.2 Maternal popliteal lymph node cellular and humoral responses to foetal or paternal cells. Pregnant ewes were injected with:

(a) 0.6×10^8 foetal thoracic duct cells at day 116,
(b) 0.6×10^8 foetal thoracic duct cells at day 118,
(c) 1.0×10^8 foetal thoracic duct cells at day 112,
(d) 1.5×10^8 foetal thoracic duct cells at day 122,
(e) 0.6×10^8 paternal PBL at day 97,
(f) 0.6×10^8 paternal PBL at day 93,
(g) 0.6×10^8 paternal PBL at day 96,
(h) 1.0×10^8 paternal efferent lymphatic cells at day 124.

Single continuous line indicates total cell output. Hatched area represents the blast cell response. Representative results of cytotoxic antibody titration are indicated by the interrupted line in (a) and (b).


(a)
(b)
(c)
(d)
25
20
15
10
5
0
5
10
15
Cell output (X10^{-7}/HR)
Log$_2$Ab titre
2
3
4
5
DAYS AFTER CHALLENGE


(e)
(f)
(g)
(h)
Cell output (X10 7 /HR)
25
20
15
10
5
0
5
10
15
DAYS AFTER CHALLENGE

lymphocytes in response to from 2.0 to 2.5 x 10^8 allogeneic cells, and the peak output occurred on the 5th to 7th day after stimulation to be followed by the appearance of large cells (Figure 2.1). The latter were typical blast cells with markedly basophilic cytoplasm and a large nucleus with several nucleoli and fine reticular chromatin,characteristics of transformed lymphocytes (Bessis 1977). The maximal total cell and blast cell outputs ranged from 7.5 to 22.3 x 10^6/hr and from 16.5 to 27.9 x 10^5/hr respectively. The pattern of response of pregnant ewes generally resembled that of control animals regardless of the dose and origin of the cells used for challenge. Even animals which had received as few as 6 x 10^7 foetal thoracic duct (Figure 2.2-(a) and (b)) or 6 x 10^7 paternal (Figure 2.2-(e), (f) and (g)) cells responded as well as non-pregnant ewes which were in receipt of from 2.0 to 2.5 x 10^8 allogeneic cells (Figure 2.1). Maximal total cell and blast cell outputs from pregnant ewes ranged from 7.8 to 22.7 x 10^6/hr and from 5.7 to 48.2 x 10^5/hr respectively.

b) Cytotoxic Antibody Response

The titre of cytotoxic antibody in lymph was determined as described in Materials and Methods. As shown in Table 2.2, 8 out of 10 pregnant ewes produced cytotoxic antibody in response to the injection of either foetal or paternal cells. Cytotoxic antibody could be detected from 7 days after challenge and peaked around the 10th day. Representative results are shown in Figure 2.2-(a) and (b). Blockage of popliteal cannulae on the 6th day precluded subsequent collection of lymph in the two animals in which antibody was not detected.

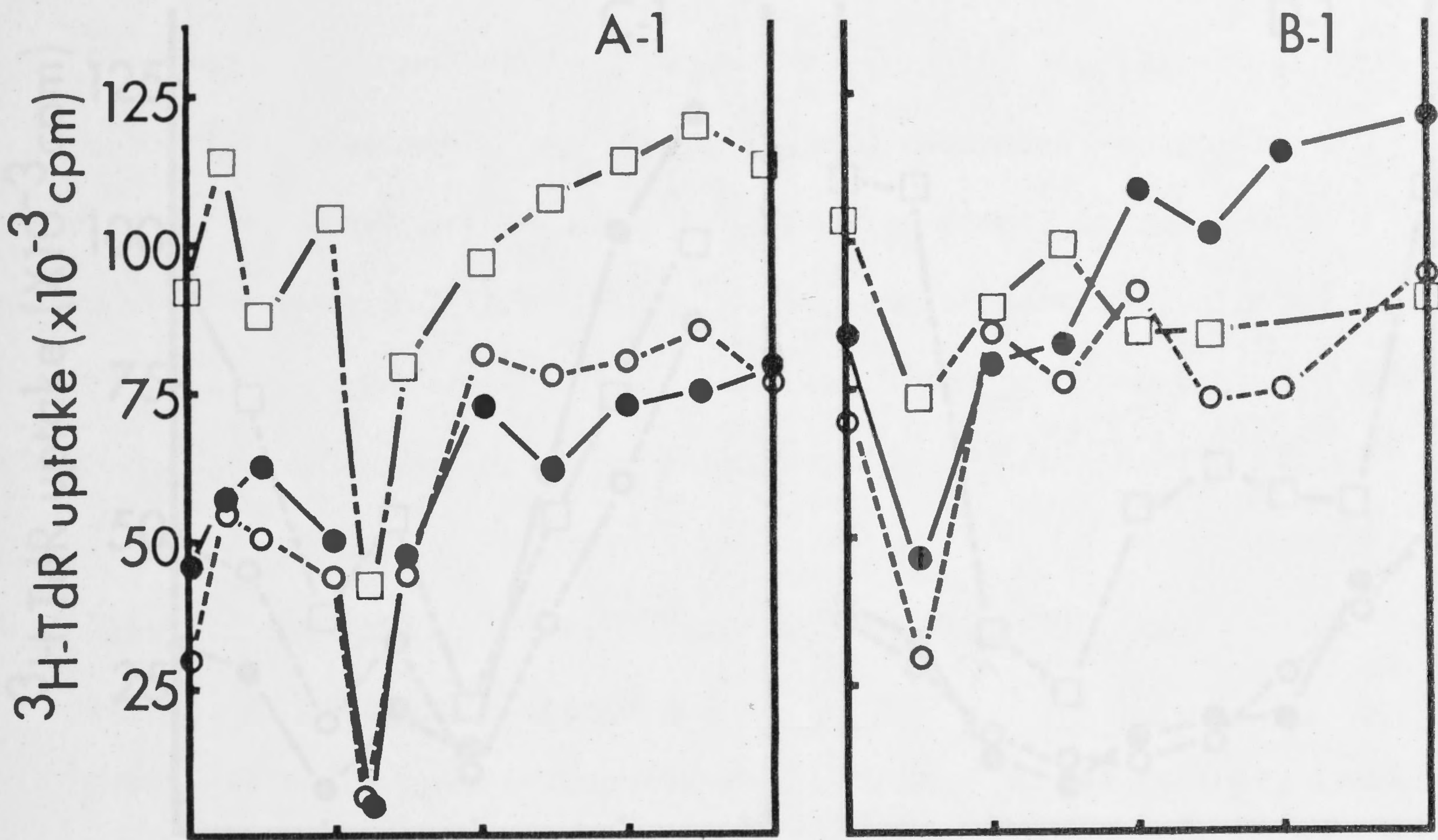
c) MLC Reactivity of Efferent Lymphocytes from Pregnant Ewes before and after Challenge of the Regional Lymph Node with Paternal or Foetal Lymphocytes

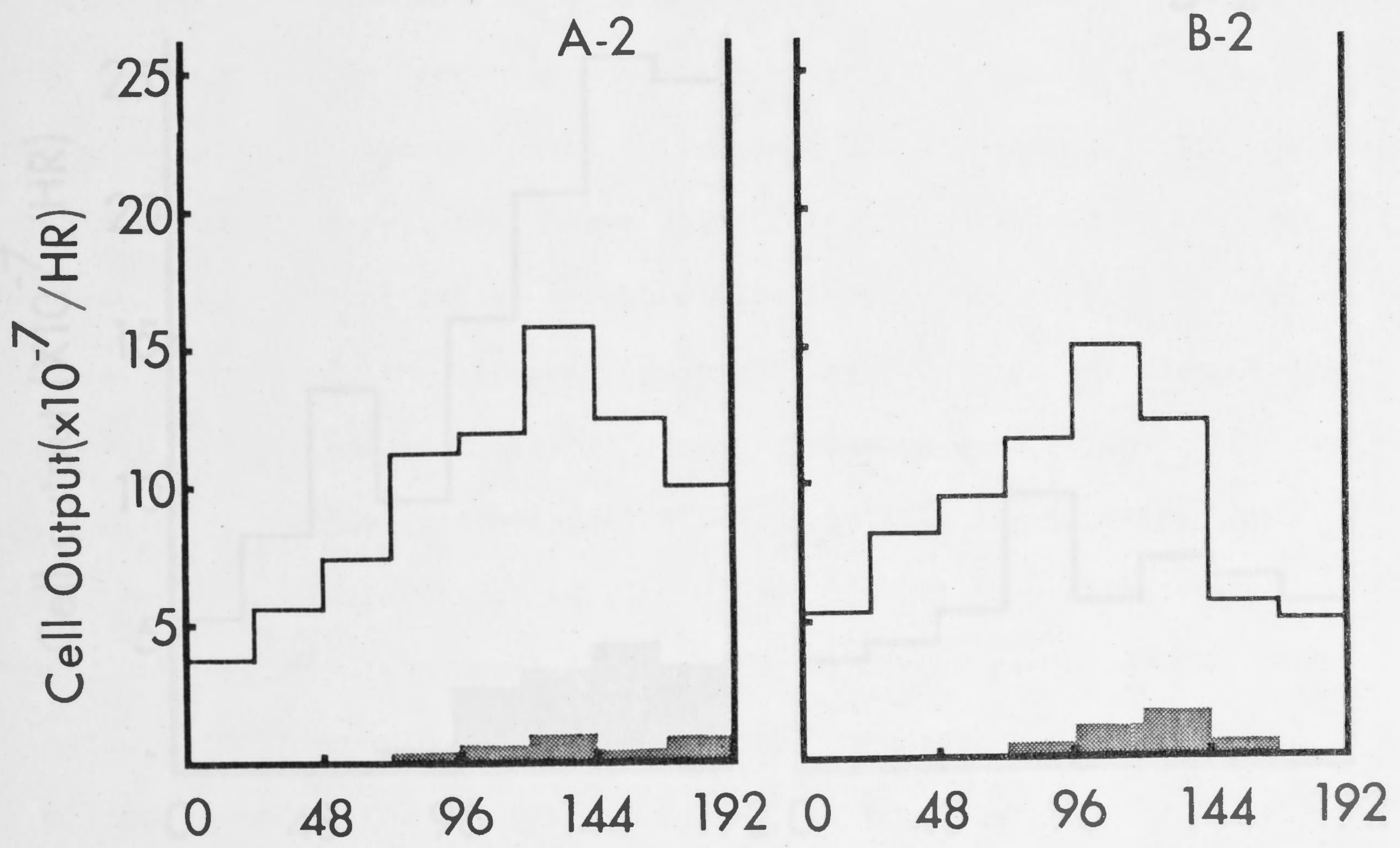
It was shown in the preceding section that administration of foetal or paternal lymphocytes markedly increased the lymphocyte traffic in the

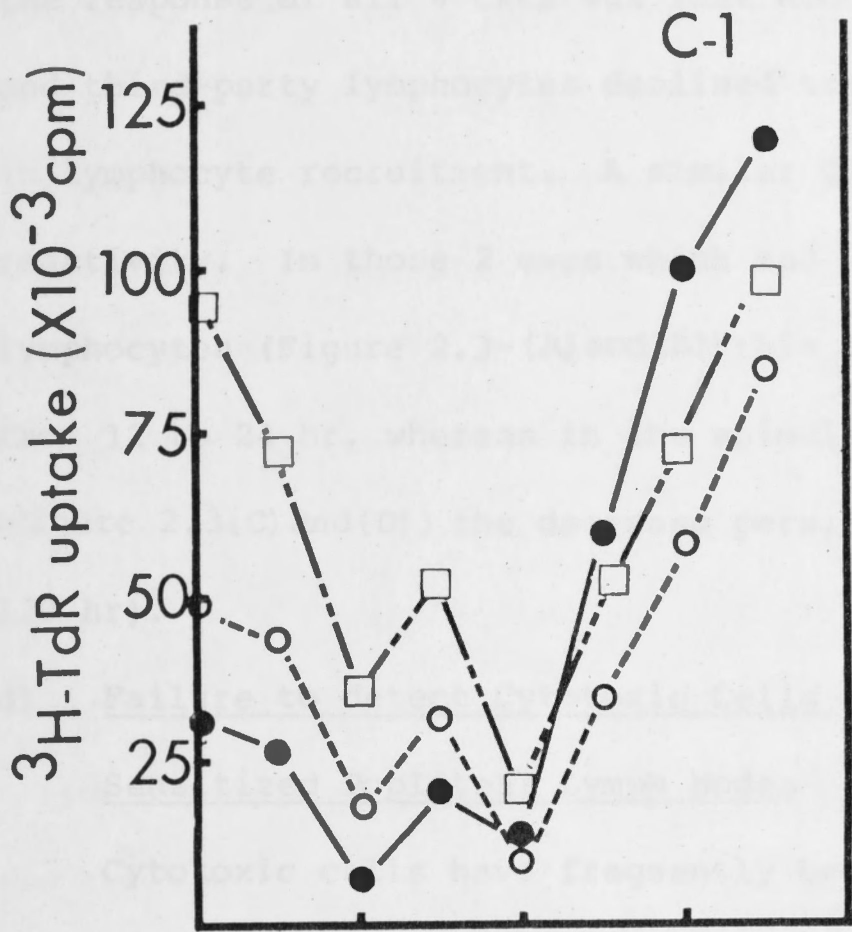
maternal regional lymph node. An increased lymphocyte traffic has been observed in all instances in which the immune response of a single lymph node has been studied (Hall and Morris 1963, Hall, 1967, Smith, Pearson and Morris 1970, Pederson and Morris 1970, McConnell, Lachmann and Hobart, 1974, Hay, Cahill and Trnka 1974a, 1974b, Cahill et al. 1979). Morris (1968) suggested that the increased traffic results from an increase in the recruitment of lymphocytes from the blood into the stimulated node because the emerging cells had neither been formed within the node nor derived from the afferent lymphatics. An interesting feature of this phenomenon is that it occurs well before transformed basophilic cells appear in the efferent lymph. Hay, Cahill, and Trnka (1974a) and Cahill et al. (1979) observed a profound decrease in specific antigen reactivity of the cells in the efferent lymph draining from the immunized node over the period of lymphocyte recruitment. They speculated that the selective removal of specifically reactive cells from the recirculatory pool was the responsible mechanism.

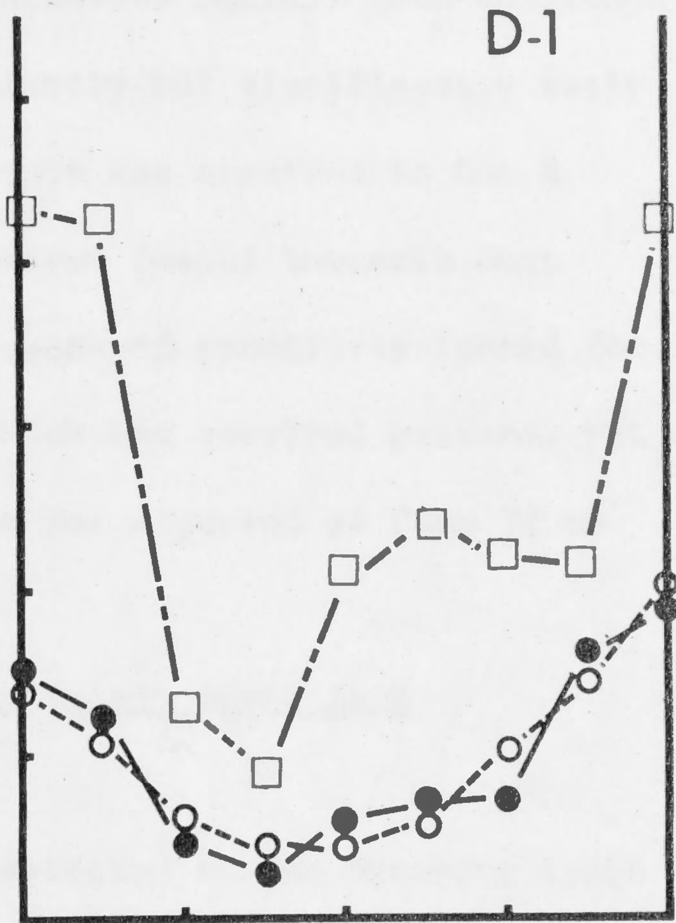
In the current investigation, cells specifically reactive against paternal and third-party cells were assayed, both before and at intervals after, stimulation of the maternal popliteal lymph node with either foetal or paternal lymphocytes using an approach similar to that adopted by Hay and others. (Hay, Cahill and Trnka 1974a, Cahill et al. 1979). Of the 4 animals used in this study, 2 were stimulated with 0.6×10^8 foetal thoracic duct cells at 116 and 118 days gestation and the remaining 2 were stimulated with the same number of paternal PBL at 93 and 96 days. Efferent cells, collected before stimulation and every 12 to 24 hrs afterwards, were cryopreserved. All frozen cells from each individual ewe were simultaneously thawed so that their alloreactivity against paternal and third-party cells in MLC could be compared in the same assay batch. The results are shown in Figure 2.3. MLC responses are shown in the upper graphs (A-1, B-1, C-1, D-1) and total cell output and blast cell response are shown in the lower

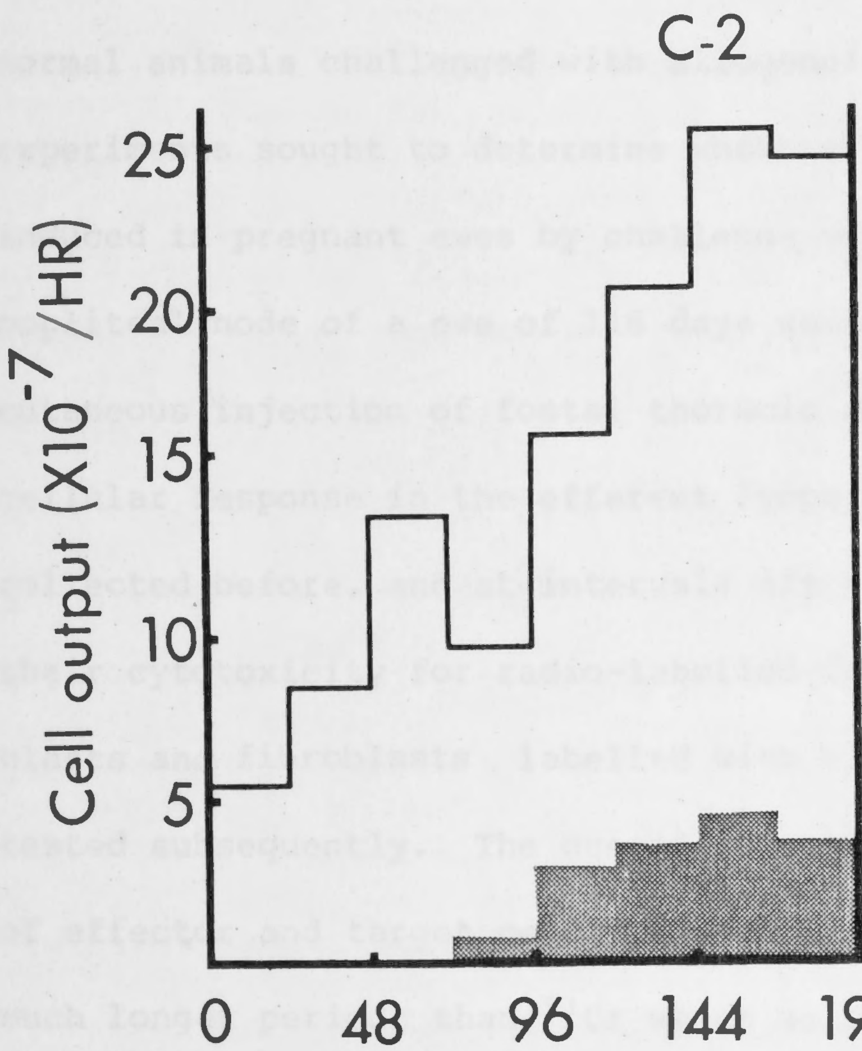
Figure 2.3 Maternal popliteal lymph node response to 0.6×10^8 foetal cells (A,B) and 0.6×10^8 paternal cells (C,D). Upper graphs show changes of MLC reactivity against foetal (A,B) or paternal (C,D) cells (●) and against third-party cells (○) and changes in the Con A reactivity (□) of the recipient efferent lymphatic cells. The lower graphs show total cell output (solid line) and blast cell response (hatched area) in the lymph draining from the regional lymph node.

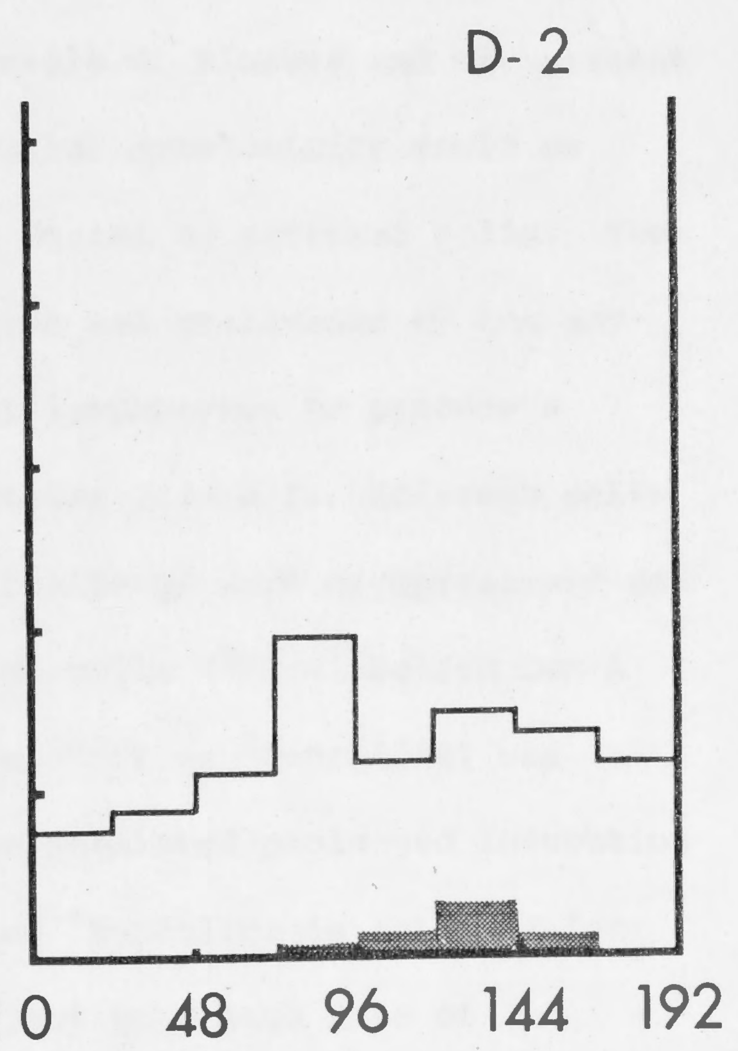

A-1
B-1
125
100
75
50
25
^{3}H-TdR uptake (x10^{-3}cpm)


A-2
B-2
25
20
15
10
5
Cell Output(x10^{-7}/HR)
0
48
96
144
192
0
48
96
144
192
HOURS AFTER CHALLENGE









HOURS AFTER CHALLENGE

graphs (A-2, B-2, C-2, D-2). There was always a certain level of MLC reactivity before challenge, presumably reflecting the histocompatibility difference between maternal and paternal animals. A prominent feature of the response of all 4 ewes was that MLC reactivity against both challenge and third-party lymphocytes declined transiently but significantly early in lymphocyte recruitment. A similar decrease was observed in Con A reactivity. In those 2 ewes which had received foetal thoracic duct lymphocytes (Figure 2.3-(A) and (B)) this decrease of reactivity lasted for from 12 to 24 hr, whereas in the animals which had received paternal PBL (Figure 2.3(C) and (D)) the decrease persisted for a period of from 72 to 120 hr).

d) <u>Failure to detect Cytotoxic Cells in Efferent Lymph from Sensitized Popliteal Lymph Nodes</u>

Cytotoxic cells have frequently been detected in the draining lymph nodes (Canty and Wunderlich 1971) and spleen (Brunner et al. 1970) of normal animals challenged with allogeneic cells or tissues and the present experiments sought to determine whether similar cytotoxicity could be induced in pregnant ewes by challenge with foetal or paternal cells. The popliteal node of a ewe of 116 days gestation was challenged by the subcutaneous injection of foetal thoracic duct lymphocytes to produce a cellular response in the efferent lymph (Figure 2.2-(a)). Efferent cells collected before, and at intervals after, challenge were cryopreserved and their cytotoxicity for radio-labelled foetal cells (^{51}Cr-labelled Con A blasts and fibroblasts labelled with either ^{51}Cr or ^{3}H-proline) was tested subsequently. The use of ^{3}H-proline permitted prolonged incubation of effector and target cells as incorporated ^{3}H-proline is retained for much longer periods than ^{51}Cr which is subject to a high rate of spontaneous release. PHA and Con A blasts were obtained by incubating foetal lymphocytes with the appropriate mitogen and fibroblasts were harvested from cultures of skin derived from the foetus. The assay period

was 6 hr in the case of ^{51}Cr assay and 20 hr for ^{3}H-proline and the effector-target ratio was 100 : 1. As evidenced by Table 2.3, cytotoxicity was not observed in sensitized cells collected during the first 240 hr after challenge. It has been demonstrated (Wagner et al. 1976) that in vitro incubation of sensitized lymphocytes which are marginally cytotoxic may significantly augment their cytotoxicity. Accordingly, lymphocytes collected after challenge were cultured in vitro for 60 hr, both in the presence and the absence of irradiated foetal lymphocytes, and then washed, re-adjusted in number and incorporated in a cytotoxicity test. However, there was no indication of cytotoxic activity as a result of this modification (Table 2.3 - II and III).

To determine whether the inability to evoke a cell-mediated cytotoxic response could be related to the pregnant state, the preceding experiment was repeated using a non-pregnant ewe. The animal was challenged with cells from an unrelated donor the histocompatibility of which had been established by the positive mixed lymphocyte reaction observed between recipient cells collected before challenge and irradiated donor cells (50,235 ± 540 cpm/1,202 ± 153 cpm; autologous control). The cellular response to challenge was as shown in Figure 2.1-(a) but cytotoxicity was not observed (Table 2.4).

In response to the preceding negative results, attempts were made to detect newly generated cytotoxic cells in the course of a primary MLC. Either efferent cells or PBL from a normal ewe were cultivated with irradiated allogeneic PBL and then tested for cytotoxic activity against ^{51}Cr-labelled Con A blasts derived from the immunizing animal as described by Granberg (1980). Although good MLC responses were obtained, the observed cytotoxicity was negligibly small (Figure 2.4). In another similar experiment (data not shown), extension of the assay period to 20 hr failed to reveal any increase in cytotoxicity. Clearly, the failure of cytotoxic cells to become evident in response to challenge of pregnant

Table 2.3 Lack of cytotoxic activity of maternal efferent lymph cells sensitized against foetal determinants.

	Time of collection of cells after challenge (hr)	Preliminary treatment of maternal lymphocytes	% Cytotoxicity 1)		
			^{51}Cr-Con A blasts	^{51}Cr-fibroblasts	^{3}H-proline fibroblasts
I	0	None	-10.7	-0.5	4.4
	72		n.d.	n.d.	-0.2
	96		- 9.7	-1.4	n.d.
	144		- 7.0	-1.6	-0.7
	168		- 8.8	-2.0	n.d.
	240		- 9.7	-2.0	-0.3
II	0	Cultured for 60 hr	+ 8.6		
	72		n.d.		
	96		- 3.4	n.d.[2]	n.d.
	144		- 6.1		
	168		- 7.3		
	240		- 5.3		
III	0	Cultured with priming cells for 60 hr	-12.8		
	72		n.d.		
	96		- 8.5		
	144		- 9.5	n.d.	n.d.
	168		-12.0	n.d.	n.d.
	240		-10.4		

1) $\% \text{ Cytotoxicity} = \frac{\text{experimental release - spontaneous release}}{\text{maximum release - spontaneous release}} \times 100$

2) Not determined.

3) Spontaneous release was 25 ∿ 30% for ^{51}Cr-labelled cells and 20 ∿ 22% for ^{3}H-proline-labelled cells.

4) Lymphocyte-target ratio was 100 : 1.

5) Incubation time was 6 hr for the ^{5}Cr release assay and 20 hr for the ^{3}H-proline assay.

Table 2.4 Non-cytotoxicity of efferent lymph cells from a non-pregnant ewe sensitized with allogeneic lymphocytes.

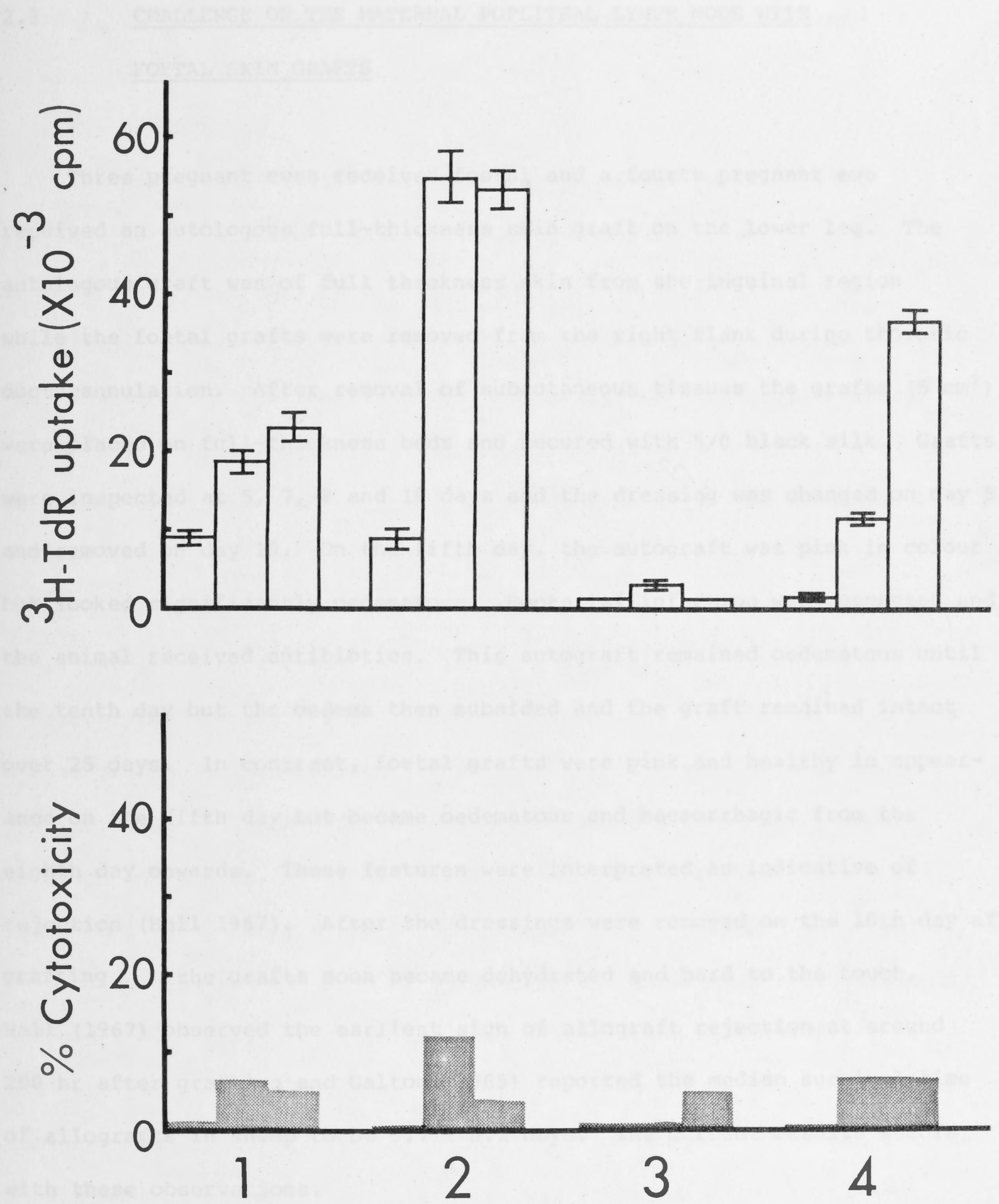
Time of collection of cells after challenge	% Cytotoxicity	
	^{51}Cr-PHA blasts	^{51}Cr-Con A blasts
0	0	0
120	+ 8.4	+ 3.9
190	+ 4.5	+ 0.6

See Legend of Table 2.2

Figure 2.4 Primary MLC responses of efferent lymphatic cells and PBL and their subsequent cytotoxic activity. Efferent lymphatic cells and PBL were collected from an unchallenged normal donor and subjected to a primary challenge in vitro. The resulting MLC responses are shown in the upper and cytotoxic activities of the cells in the lower graph. Each group of 3 columns represents mean ± S.E. on days 3, 5 and 7 in the case of MLC and mean % cytotoxicity on days 3, 5 and 7 in the cytotoxic assays. The combinations of cells used in the cultures were:

1. efferent lymphatic cells and irradiated autologous efferent lymphatic cells,
2. efferent lymphatic cells and irradiated allogeneic PBL,
3 PBL and irradiated autologous PBL,
4 PBL and irradiated allogeneic PBL.

Target cells in the cytotoxic assay were Con A blasts derived from that allogeneic animal from which the stimulator cells used in the primary MLC had been collected.



^{3}H-dR uptake (X10^{-3} cpm)
60
40
20
0
% Cytotoxicity
40
20
0
1
2
3
4

ewes is no more than an instance of the failure of such cells to be readily demonstrable in response to allogeneic challenge of the sheep.

2.3 CHALLENGE OF THE MATERNAL POPLITEAL LYMPH NODE WITH FOETAL SKIN GRAFTS

Three pregnant ewes received foetal and a fourth pregnant ewe received an autologous full-thickness skin graft on the lower leg. The autologous graft was of full thickness skin from the inguinal region while the foetal grafts were removed from the right flank during thoracic duct cannulation. After removal of subcutaneous tissues the grafts (5 cm^2) were placed on full-thickness beds and secured with 5/0 black silk. Grafts were inspected at 5, 7, 8 and 10 days and the dressing was changed on day 5 and removed on day 10. On the fifth day, the autograft was pink in colour but looked significantly oedematous. Bacterial infection was suspected and the animal received antibiotics. This autograft remained oedematous until the tenth day but the oedema then subsided and the graft remained intact over 25 days. In contrast, foetal grafts were pink and healthy in appearance on the fifth day but became oedematous and haemorrhagic from the eighth day onwards. These features were interpreted as indicative of rejection (Hall 1967). After the dressings were removed on the 10th day after grafting the grafts soon became dehydrated and hard to the touch. Hall (1967) observed the earliest sign of allograft rejection at around 200 hr after grafting and Galton (1965) reported the median survival time of allografts in sheep to be 9.7 ± 0.2 days. The current results accord with these observations.

C. DISCUSSION

In this section, an attempt was made to study the *in vivo* maternal

immune response against paternal and foetal determinants. No cytotoxic activity was detected in 104 plasma specimens obtained from 18 primigravid ewes which had not been deliberately challenged and it appears that the spontaneous occurrence of cytotoxic antibodies reported in multiparous sheep (Ford and Elves 1974) is a rare phenomenon in a first pregnancy.

The kinetics of cell output from the popliteal lymph node in response to stimulation with allogeneic lymphocytes have been thoroughly studied (Hall 1964, Hay, Cahill and Trnka 1974a, Cahill et al. 1979). Hall (1964) found the responses in efferent lymph to be monophasic and very similar to responses to particulate antigens. However, Trnka's group (Hay, Cahill and Trnka 1974a, Cahill et al. 1979) reported that the output of lymphocytes in efferent lymph was biphasic. The reason for this difference is not known. In the current study, not only the response of non-pregnant sheep to allogeneic cells but also the maternal popliteal lymph node response to foetal and paternal lymphocytes was invariably monophasic. The 'shutdown' phenomenon, a transient fall in the number of lymphocytes leaving the node in the efferent lymph during the initial stage of lymph node responses to a variety of antigens (Smith, Pederson and Morris 1970), did not occur constantly in the present study, being observed in only 6 out of 13 instances. Ten pregnant ewes, which were between 93 and 127 days of pregnancy, all manifested a significantly increased lymphocyte output of varying degrees from the stimulated node and this response was followed by an increase in the basophilic lymphoblast content of the efferent lymph. A cytotoxic antibody response was detected in all ewes in which the popliteal cannula remained patent for a week. This cytotoxic antibody was not characterized immunochemically.

When the anti-paternal or anti-foetal MLC reactivity of cells from the efferent lymph of the stimulated node was assayed, both of these activities and also the responsiveness against Con A were severely depressed

when efflux of lymphocytes from the node was increasing. The non-specificity of this depression of reactivity is at variance with previous reports (Hay, Cahill and Trnka 1974a, Cahill et al. 1979) of a short-lived, but specific, depletion of MLC reactivity accompanied by retention of full PPD reactivity, in efferent lymph cells after challenge of a popliteal node with allogeneic lymphocytes. This led these workers to conclude that the selective removal of specific lymphocytes from the recirculating pool had occurred. Similar observations that antigenic challenge of an animal can temporarily impair the capacity of its circulating lymphocytes to respond to the priming antigen after re-exposure have been reported by others (Sprent, Miller, Mitchell 1971, Ford and Atkins 1972, Rowley et al. 1972, Larner 1973, McCullagh 1973, 1975c, 1977, 1980a, 1980b). The reduced reactivity has been interpreted as a consequence of the selective removal of antigen reactive cells from the circulation although an alternative explanation has been proposed on the basis of:

(1) the observed reactivation of unreactive populations following their removal from the antigenically challenged animals, and

(2) the capacity of unreactive populations to modify the reactivity of normal populations of lymphocytes if they are transferred together to irradiated hosts (McCullagh 1980a).

Differences in experimental design between the current investigation and the experiments of Hay, Cahill and Trnka (1974a) and Cahill et al. (1979) arose in the dose and source of challenging cells and in the status of the recipient. In the earlier study 2 x 10^8 cells from normal allogeneic donors were injected into non-pregnant recipients whereas the ewes in the present experiments received from 0.6 to 1.5 x 10^8 paternal or foetal cells. The concurrent reduction of MLC reactivity against third-party cells in these experiments is unlikely to have reflected a sharing of specificity between paternal and third-party sheep as the decrease in Con A reactivity indicates that nonspecific depression had occurred. MLC and Con A responses

are mediated by surface immunoglobulin-negative lymphocytes in sheep (Cahill et al. 1978). While it remains possible that the decrease of MLC and Con A reactivities resulted from partial replacement of these cells by other lymphocyte subpopulations, the MLC dose/response studies reported in Chapter 1 indicate that a very marked reduction in the numbers of responder cells would have been required. Other possible mechanisms for the depressed reactivity after challenge could be a nonspecific humoral inhibitory factor similar to that observed in rats immediately after antigenic challenge (McCullagh 1980a) or suppressive cells (McCullagh 1975c, 1977). Lymphocytes from foetal or newborn animals have been reported to secrete molecules which can inhibit proliferation of maternal lymphocytes (Olding, Murgita, Wigzell, 1977). Although it is conceivable that molecules secreted by injected foetal cells transiently impaired the maternal response, the efficacy with which paternal lymphocytes also lowered the reactivity of efferent cells does not support this explanation.

Attempts to detect cytotoxic activity on the part of cells efferent from a maternal popliteal node challenged with foetal cells were unsuccessful. Grant and Cameron (1975), using ^{51}Cr-labelled small lymphocytes (not blasts) as target cells, demonstrated weak cytotoxicity on the part of lymphocytes efferent from the popliteal node sensitized with allogeneic lymphocytes. However, the significance of this observation is not clear because of the very high effector-target ratio (150 : 1) and long assay period (20 hr) adopted. Unsuccessful attempts were made to generate cytotoxic cells in vitro. The method used was that of Granberg (1980) who successfully generated cytotoxic cells by culturing sheep PBL with irradiated allogeneic PBL for 5 days. Detectable cytotoxicity did not exceed 15% in the present study whereas Granberg reported levels of from 50 to 90%. Foetal fibroblasts, in addition to the Con A blasts used by Granberg, were employed as a source of target cells in the

present study. The spontaneous release of radioisotope from fibroblasts was considerably less than that from Con A blasts and this permitted the use of a longer incubation time of target with effector cells. However, increased cytotoxicity was not observed even with prolongation of the assay time to 20 hr (fibroblasts incorporated from 0.1 to 1.2 cpm of radioactivity per cell which was considered satisfactory for an assay of this type (Brunner, Engers and Cerottini 1976)).

Although considerable variation exists in the sensitivity of different target cells to lysis, mitogen-induced lymphoblasts have been regarded as sensitive target cells (Brunner, Engers and Cerottini 1976) and Granberg (1980) has been successful in lysing sheep Con A blasts with cytotoxic cells generated in vitro. The cultivation conditions of lymphocytes in the present experiments seemed to be appropriate for supporting cell division of lymphocytes judging from the vigorous proliferation of cells in MLC and mitogen-induced blastogenesis. However, the possibility exists that culture conditions, although favourable for proliferation of MLC reactive cells, were not suitable for the induction of cytotoxic cells in vitro.

All foetal skin grafts were rejected by mothers within 8 to 10 days. In contrast, an autograft survived indefinitely. Although there is only one experimental autograft available in the current study, this corresponds to the earlier studies. Hall (1967) observed successful full-thickness autografts in 4 out of 4 animals and Galton (1965) did not have any failure of full-thickness autografts in 16 sheep. These results suggest that the maternal lymphoid system is capable of recognizing and responding to foetal or paternal cells and tissues and eliminating them. This is in accord with the report of Galton (1965) to the effect that full-thickness ear skin grafts from 10 to 15 week old lambs were rejected at a typical first set rate by ewes. Similarly, pregnancy did not alter the

course of rejection of allografts applied between 203 and 249 days of pregnancy to cattle which possess a similar type of placenta to sheep (Billingham and Lampkin 1957).

CHAPTER 3

THE CONSEQUENCES OF EXPOSURE OF THE FOETUS TO MATERNAL CELLS AT AN EARLY AGE

A. INTRODUCTION

The question of whether an animal carries the genetic information required to enable its immune system to recognize self antigenic determinants or whether this ability to recognize 'self' is acquired during embryogenesis remains unresolved. Unconfirmed experiments in which the posterior pituitary was removed from tree frog larvae at an early stage of development and reimplanted later suggested that exposure of the developing animal to its own tissues is essential if these are to be recognized as 'self' subsequently (Triplett 1962).

The foetus may encounter intrinsic (its own antigenic determinants) and extrinsic antigenic stimuli (probably of maternal origin). However, the extent to which early exposure of the foetus to these stimuli modifies its ability to respond to antigens in later life is unclear. Attempts to differentiate between the impact of genetic and environmental maternal influences on development of the immune system have rarely been undertaken. The present experiments examined the effects of artificial exposure of the foetal immune system to maternal cells.

B. RESULTS

Twenty-two ewes of known gestational age were used (Table 3.1). Determination of foetal age was as described in Materials and Methods. In the present experiments, extreme attention was directed to maintenance of sterility and minimization of surgical trauma. Ewes were treated with penicillin and streptomycin for three days after surgery. In the 9 animals which received bone marrow cells, both uterine wall and foetal membranes were incised to locate the foetus and subsequently to administer the injection accurately into the peritoneal cavity. This procedure could be completed with the loss of only small amounts of amniotic fluid. In the

Table 3.1 Consequences of exposure of young foetuses to maternal cells.

Recipient	Stage of gestation (days)	No. of cells transferred ($x 10^{-8}$)	Consequences
A. Allogeneic bone marrow cell transfer			
31F	70	5.0	Thoracic duct cannulation was performed on day 127 and the reactivity of thoracic duct cells was tested. Subsequently 6.0 x 10^9 maternal sensitized cells were injected i.v. into the foetus on day 136 and the foetus died several hours after the injection.
33F	73	2.0	Born
36F	73	2.0	Thoracic duct cannulation was performed on day 125 and the reactivity of thoracic duct cells was tested. The foetus was born prematurely on day 135 and died on the day of birth.
B. Maternal bone marrow cell transfer			
49F	50	2.0	Resorption*
47F	58	2.0	Resorption*
44F	59	1.8	Born
46F	68	4.0	Resorption*
48F	68	6.0	Resorption*
42F	70	3.0	Resorption*
C. Maternal lymph node cell transfer			
53F	43	0.5	Abortion at day 60
51F	49	1.0	Born
62F	52	0.6	Resorption*
54F	56	0.8	Born
61F	46	1.5	Resorption*
70F	48	1.5	Resorption*
71F	48	3.0	Resorption*

Table 3.1 (Contd.)

Recipient	Stage of gestation (days)	No. of cells transferred ($\times 10^{-8}$)	Consequences
68F	49	1.5	Resorption*
69F	49	1.4	Resorption*
64F	50	2.2	Resorption*
72F	51	1.8	Abortion at day 110
73F	52	1.8	Resorption*
67F	55	1.8	Resorption*

* Resorption was presumed because of failure to observe abortion.

remaining 13 foetuses, the uterine wall alone was incised using electrocautery, the foetal membranes being left intact. The foetus was visualized by transillumination through its membranes and a 28-gauge needle was introduced into the amniotic cavity through the uterine wall and then through the abdominal wall of the foetus. In most cases injected lymph node cell suspensions were seen to disperse in the foetal peritoneal cavity.

3.1 INJECTION OF ALLOGENEIC BONE MARROW CELLS

Bone marrow was aspirated from iliac crests and made into a single cell suspension. Three foetuses of from 70 to 73 days gestation received allogeneic bone marrow cells while six foetuses between 50 and 70 days received maternal bone marrow cells. All injections were given intraperitoneally. However, because of the difficulty in obtaining single cell suspensions, in preparing cell suspensions in volumes sufficiently small for injection and also in avoiding contamination with large numbers of red blood cells, lymph node cells were used in the later experiments. Thirteen foetuses from 43 to 70 days gestation received intraperitoneal injections of maternal lymph node cells. All ewes were found to carry a single foetus.

As Silverstein, Prendergast and Kraner (1964) have shown that foetal lambs of 75 days of gestational age are capable of rejecting orthotopic skin grafts, animals of this age were initially investigated. Three foetuses between 70 and 73 days gestation (31F, 33F, 36F) were injected intraperitoneally with from 2.0 to 5.0 x 10^8 allogeneic bone marrow cells. Two foetuses (31F and 36F) were submitted to thoracic duct cannulation after 125 and 127 days *in utero* to provide foetal lymphocytes whilst the third (33F) was born normally after a gestation of 148 days.

Mixed lymphocyte cultures were set up using thoracic duct lymphocytes from 31F and 36F as responder cells and their activity was tested against

PBL from the bone marrow donors and from unrelated animals. The responses of thoracic duct cells from 31F and 36F are shown in Figure 3.1 and Figure 3.2. In both cases, the peak response was observed on day 5 of culture without any suggestion of the acceleration of reactivity characteristic of sensitized cells (shown in Chapter 1). Reactivity of 31F cells against donor PBL was slightly less than reactivity against unrelated PBL but the response was still substantial (Figure 3.1). Reactivity of 36F cells against donor and third-party PBL was strong and almost identical (Figure 3.2).

On day 136, 31F received an intravenous injection of 6.0×10^9 maternal popliteal efferent lymphocytes which had been sensitized with foetal cells to test the response of the foetus to maternal immunological aggression. The foetus died immediately after the injection and the details are described in Chapter 5.

36F was born prematurely on day 135 and died on the day of birth. Fresh histology samples were not available from either of 31F or 36F but no gross abnormalities were found at autopsy.

The MLC reactivity of 33F which had received allogeneic bone marrow cells on day 73 of gestation and had then been born intact was tested at 1 week of age (Figure 3.3). The MLC response of its PBL peaked on day 7 of culture, the magnitude of response against the bone marrow donor's PBL being similar to that against third-party PBL. Addition of serum from 33F reduced the MLC response against bone-marrow donor PBL to the same extent as did sera from 2 unrelated animals (Figure 3.4). A skin graft transferred from the donor animal became very dark in colour by the 8th day and dehydrated by the 12th day which is in accord with a first-set rejection. In contrast with this normal allograft rejection, NLT reactivity of this animal was significantly depressed when this was tested 2 months after the test skin grafting (Figure 3.5). Lymphatic efferent cells were collected from the bone marrow donor and from 3 other unrelated animals.

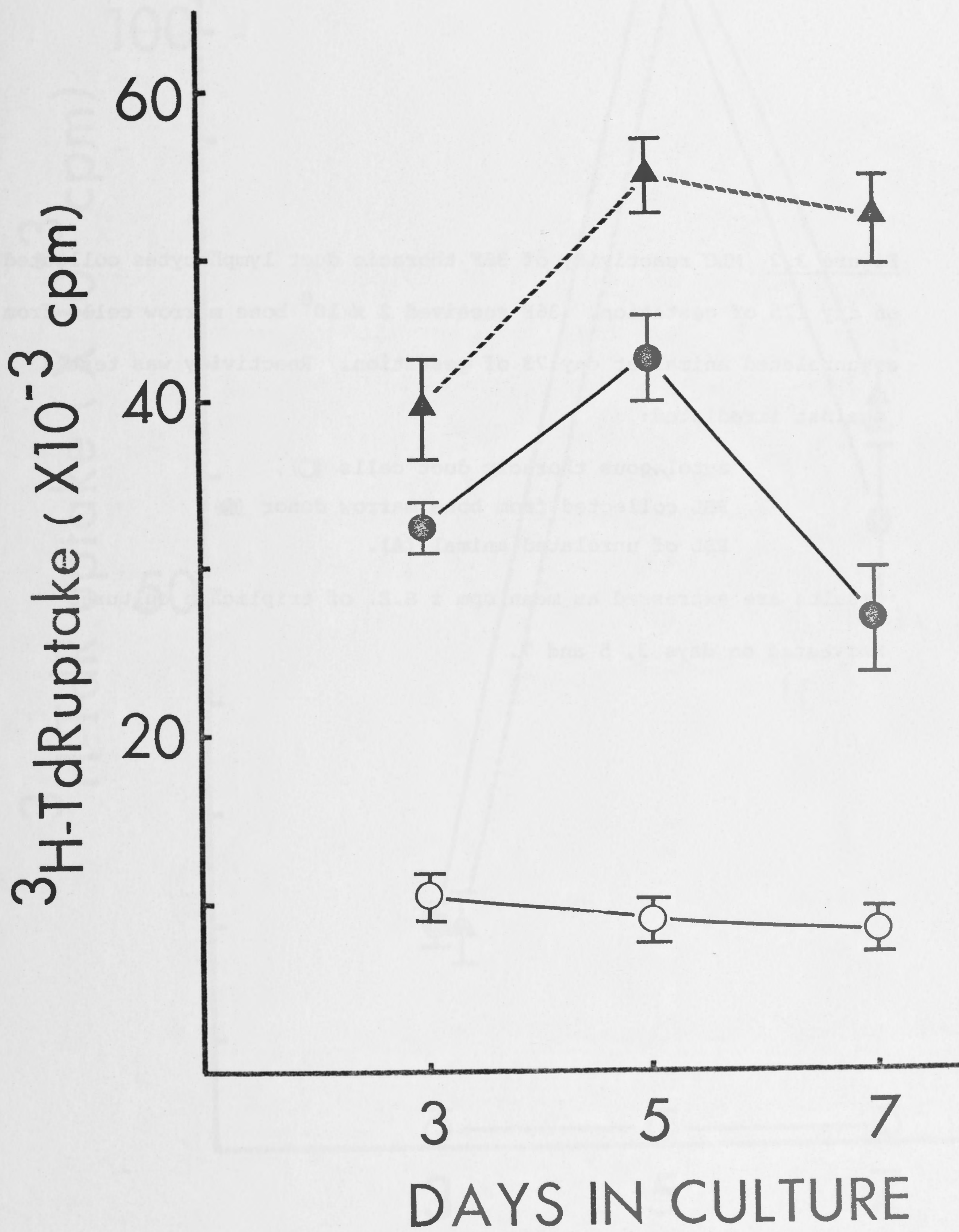
Figure 3.1 MLC reactivity of 31F thoracic duct lymphocytes collected on day 127 of gestation. 31F received 5 x 10^8 allogeneic bone marrow cells at day 70 of gestation. Reactivity of its thoracic duct lymphocytes was tested against irradiated:

autologous thoracic duct cells (○),

PBL from bone marrow donor animal (●),

PBL of unrelated animal (▲).

Results are expressed as mean cpm ± S.E. of triplicate cultures harvested on days 3, 5 and 7.

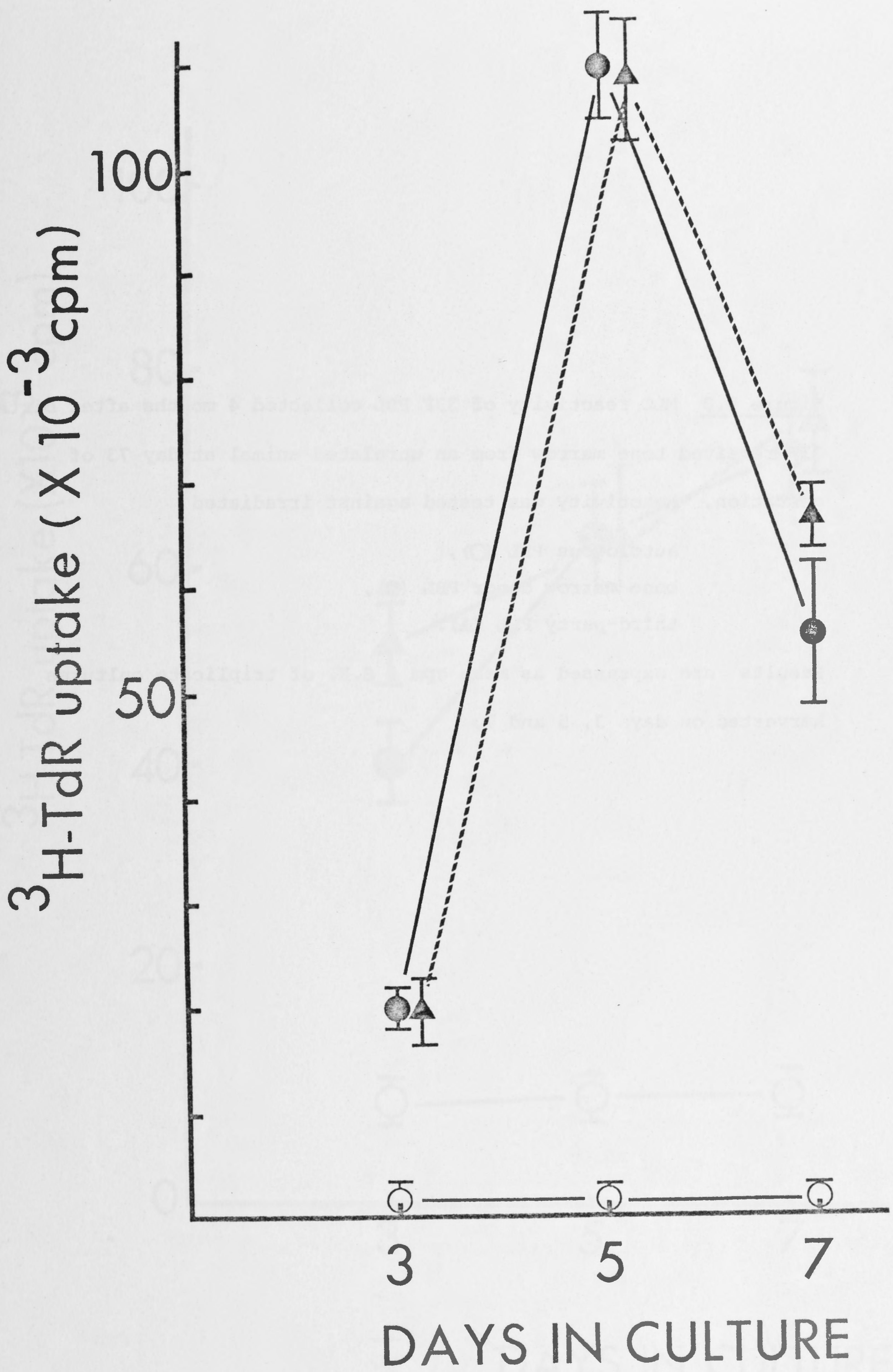


3H-TdRuptake (X10-3 cpm)
60
40
20
3
5
7
DAYS IN CULTURE

Figure 3.2 MLC reactivity of 36F thoracic duct lymphocytes collected on day 125 of gestation. 36F received 2×10^8 bone marrow cells from an unrelated animal at day 73 of gestation. Reactivity was tested against irradiated:

autologous thoracic duct cells (○),
PBL collected from bone marrow donor (●),
PBL of unrelated animal (▲).

Results are expressed as mean cpm ± S.E. of triplicate cultures Harvested on days 3, 5 and 7.



^{3}H-TdR uptake (X 10^{-3} cpm)
100
50
0
3
5
7
DAYS IN CULTURE

Figure 3.3 MLC reactivity of 33F PBL collected 4 months after birth. 33F received bone marrow from an unrelated animal at day 73 of gestation. Reactivity was tested against irradiated:

autologous PBL (○),
bone marrow donor PBL (●),
third-party PBL (▲).

Results are expressed as mean cpm ± S.E. of triplicate cultures harvested on days 3, 5 and 7.

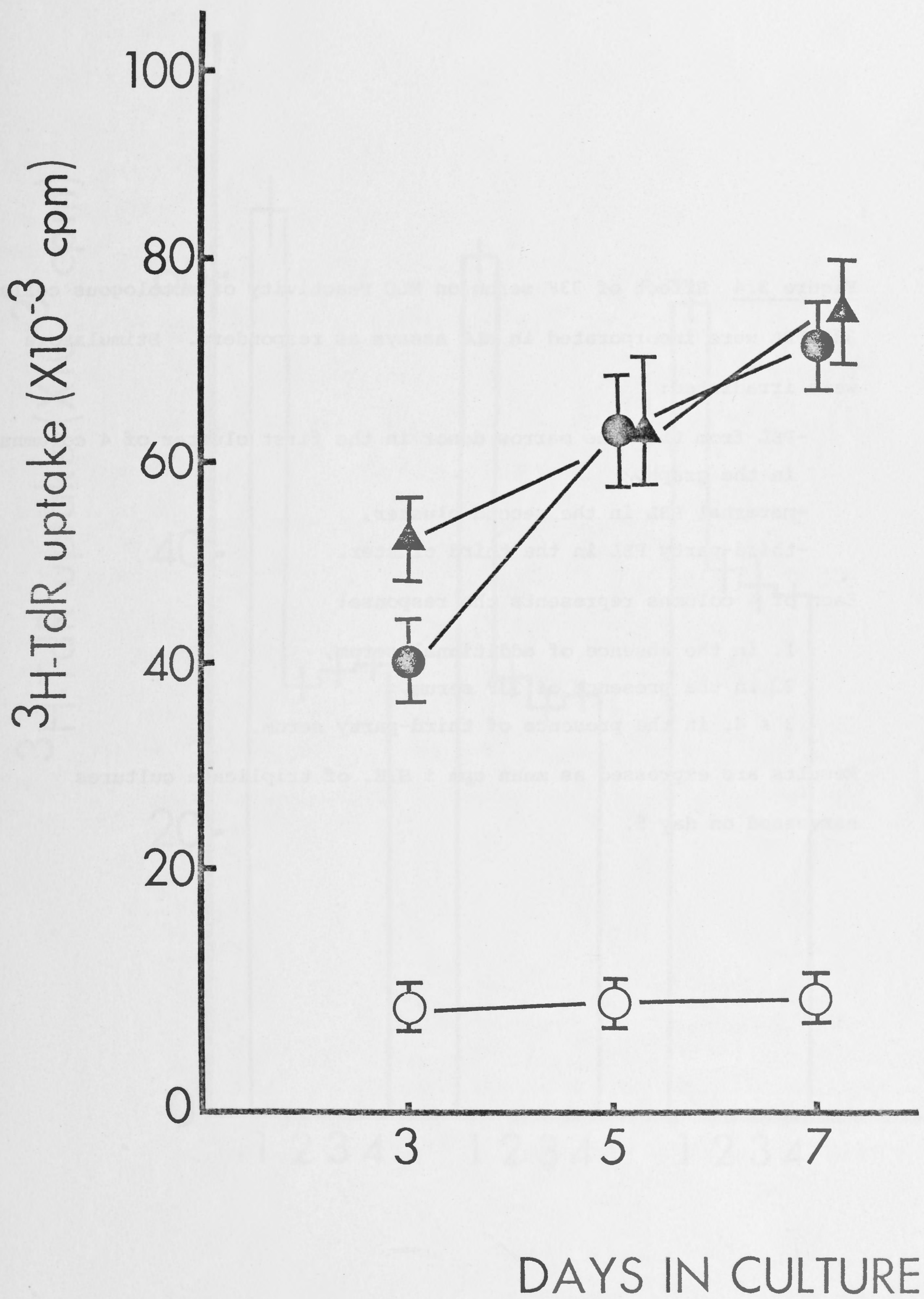

3H-TdR uptake (X10-3 cpm)
100
80
60
40
20
0
3
5
7
DAYS IN CULTURE

Figure 3.4 Effect of 33F serum on MLC reactivity of autologous cells. 33F PBL were incorporated in MLC assays as responders. Stimulators were irradiated:

-PBL from the bone marrow donor in the first cluster of 4 columns in the graph,

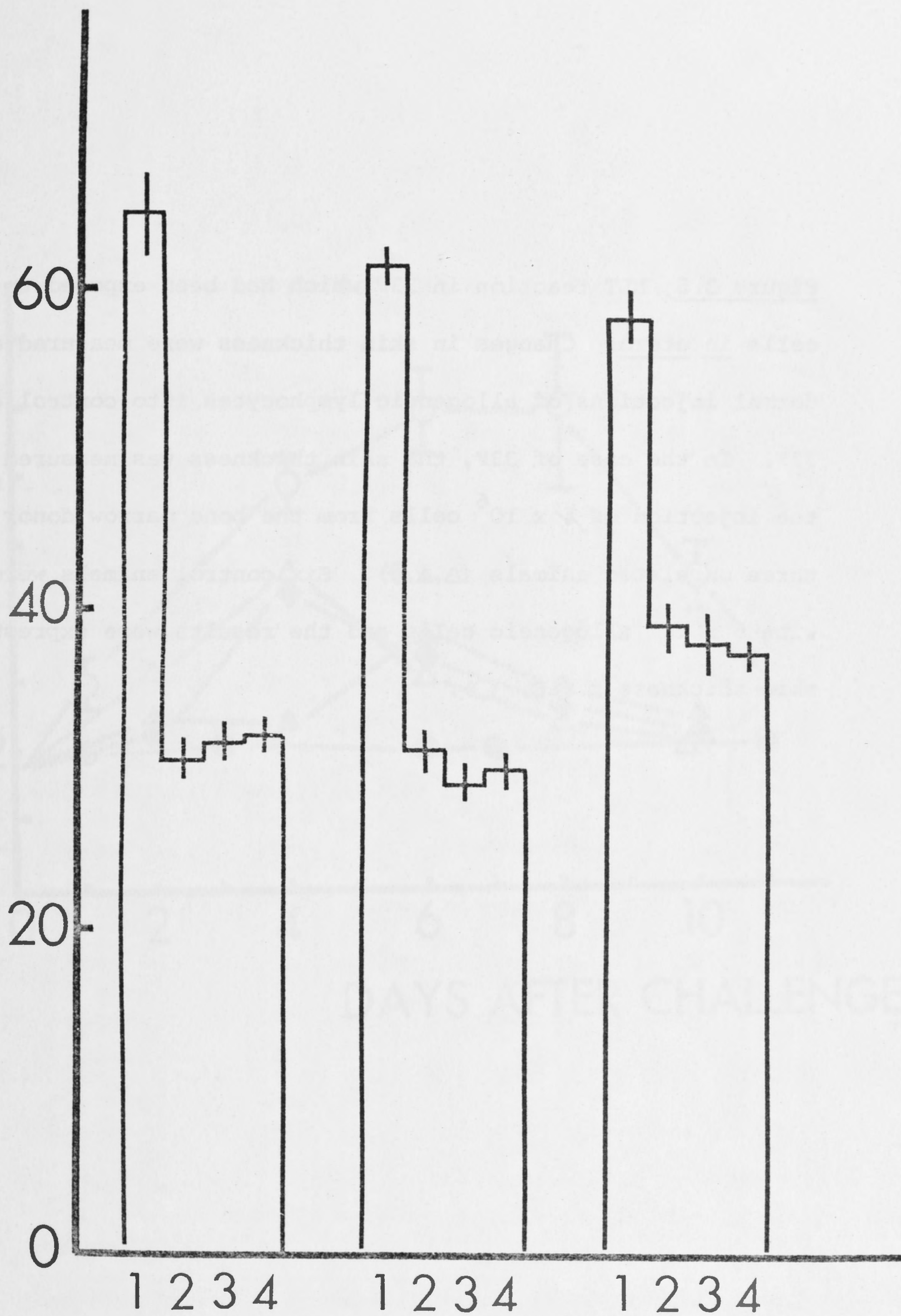
-maternal PBL in the second cluster,

-third-party PBL in the third cluster.

Each of 4 columns represents the response:

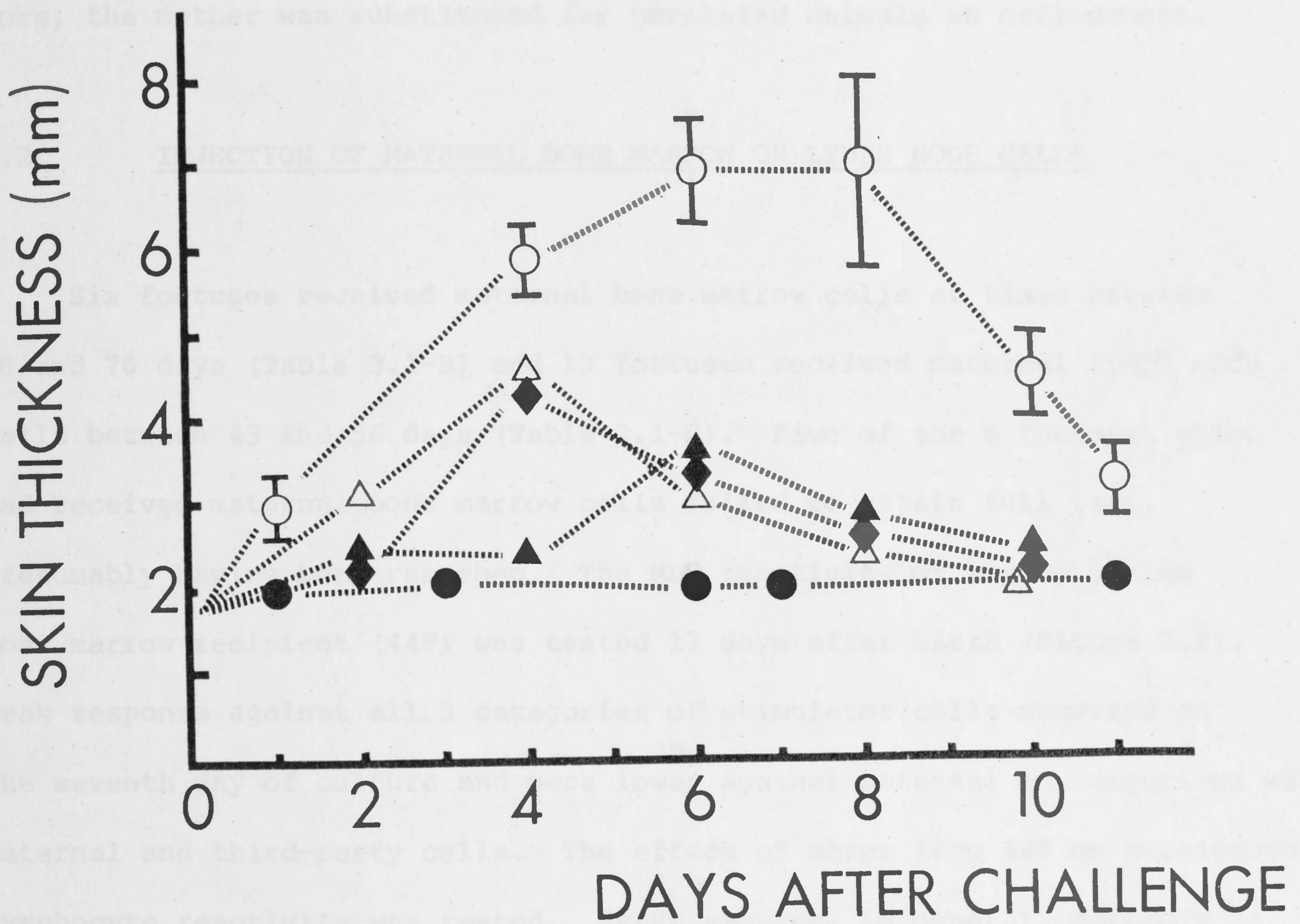
1. in the absence of additional serum,
2. in the presence of 33F serum,
3. & 4. in the presence of third-party serum.

Results are expressed as mean cpm ± S.E. of triplicate cultures harvested on day 5.



3H-TdR uptake (X10^3 cpm)
60
40
20
0
1 2 3 4
1 2 3 4
1 2 3 4

Figure 3.5 NLT reaction in 33F which had been exposed to allogeneic cells *in utero*. Changes in skin thickness were measured after intradermal injections of allogeneic lymphocytes into control animals and 33F. In the case of 33F, the skin thickness was measured following the injection of 5×10^6 cells from the bone marrow donor (●) and three unrelated animals (△,▲,◆). Six control animals were injected with 5×10^6 allogeneic cells and the results were expressed as mean skin thickness ± S.E. (○).


SKIN THICKNESS (mm)
8
6
4
2
0
2
4
6
8
10
DAYS AFTER CHALLENGE

5 x 10^6 cells from each donor were suspended in 0.1 ml Hanks' BSS and simultaneously injected intradermally into 33F at different sites. Skin thickness was measured for the next 11 days. 33F responded poorly to all 4 types of cells but the response to cells of donor origin was especially weak.

To determine whether it was possible to modify reactivity in tests other than the NLT by exposure to foreign cells in utero, a second group of foetuses were challenged at an earlier stage of development. Furthermore, the mother was substituted for unrelated animals as cell donors.

3.2 INJECTION OF MATERNAL BONE MARROW OR LYMPH NODE CELLS

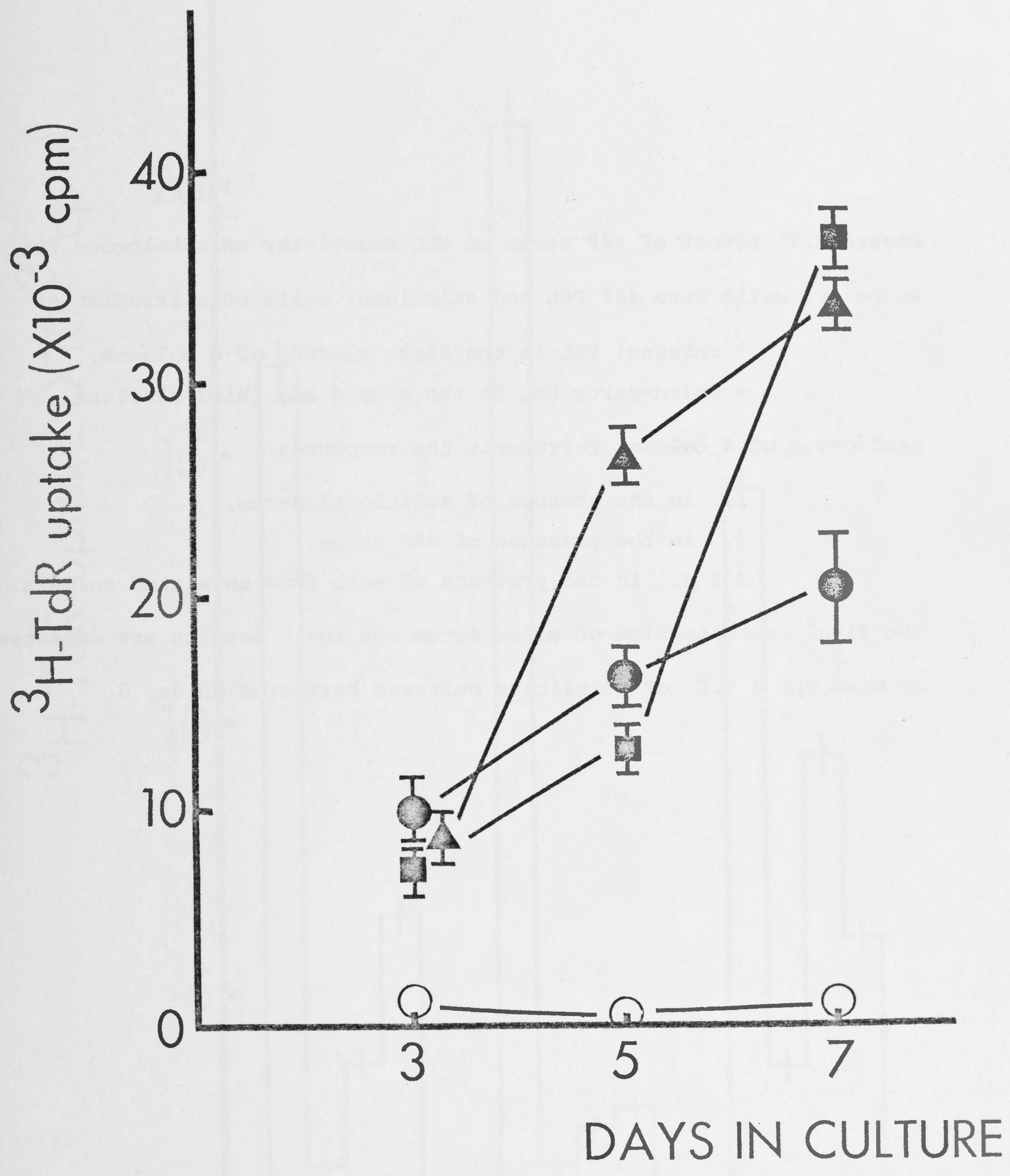
Six foetuses received maternal bone marrow cells at times between 50 and 70 days (Table 3.1-B) and 13 foetuses received maternal lymph node cells between 43 and 56 days (Table 3.1-C). Five of the 6 foetuses which had received maternal bone marrow cells failed to attain full term, presumably having been resorbed. The MLC reactivity of the surviving bone marrow recipient (44F) was tested 17 days after birth (Figure 3.6). Peak response against all 3 categories of stimulator cells occurred on the seventh day of culture and were lower against maternal in comparison with paternal and third-party cells. The effect of serum from 44F on autologous lymphocyte reactivity was tested. Serum samples, in general, markedly depressed reactivity when added to MLC. However, serum from 44F produced significantly more depression than did third-party serum samples (Figure 3.7). This depression was observed if maternal cells were used as stimulators and also in the case of one of the two batches of third-party cells used. A maternal skin graft transferred to 44F underwent rejection at normal speed.

The NLT reactivity of 44F when tested 2 months after the skin grafting was also impaired (Figure 3.8). 44F was completely unresponsive to maternal

Figure 3.6 MLC reactivity of 44F PBL collected 17 days after birth. 44F received 10^8 maternal bone marrow cells at day 59 of gestation. Reactivity was tested against irradiated:

autologous PBL (○),
maternal PBL (●),
paternal PBL (▲),
third-party PBL (■).

Results are expressed as mean ± S.E. of triplicate cultures harvested on days 3, 5 and 7.



^{3}H-TdR uptake (X10^{-3} cpm)
40
30
20
10
0
3
5
7
DAYS IN CULTURE

Figure 3.7 Effect of 44F serum on MLC reactivity of autologous PBL. Responder cells were 44F PBL and stimulator cells were irradiated:

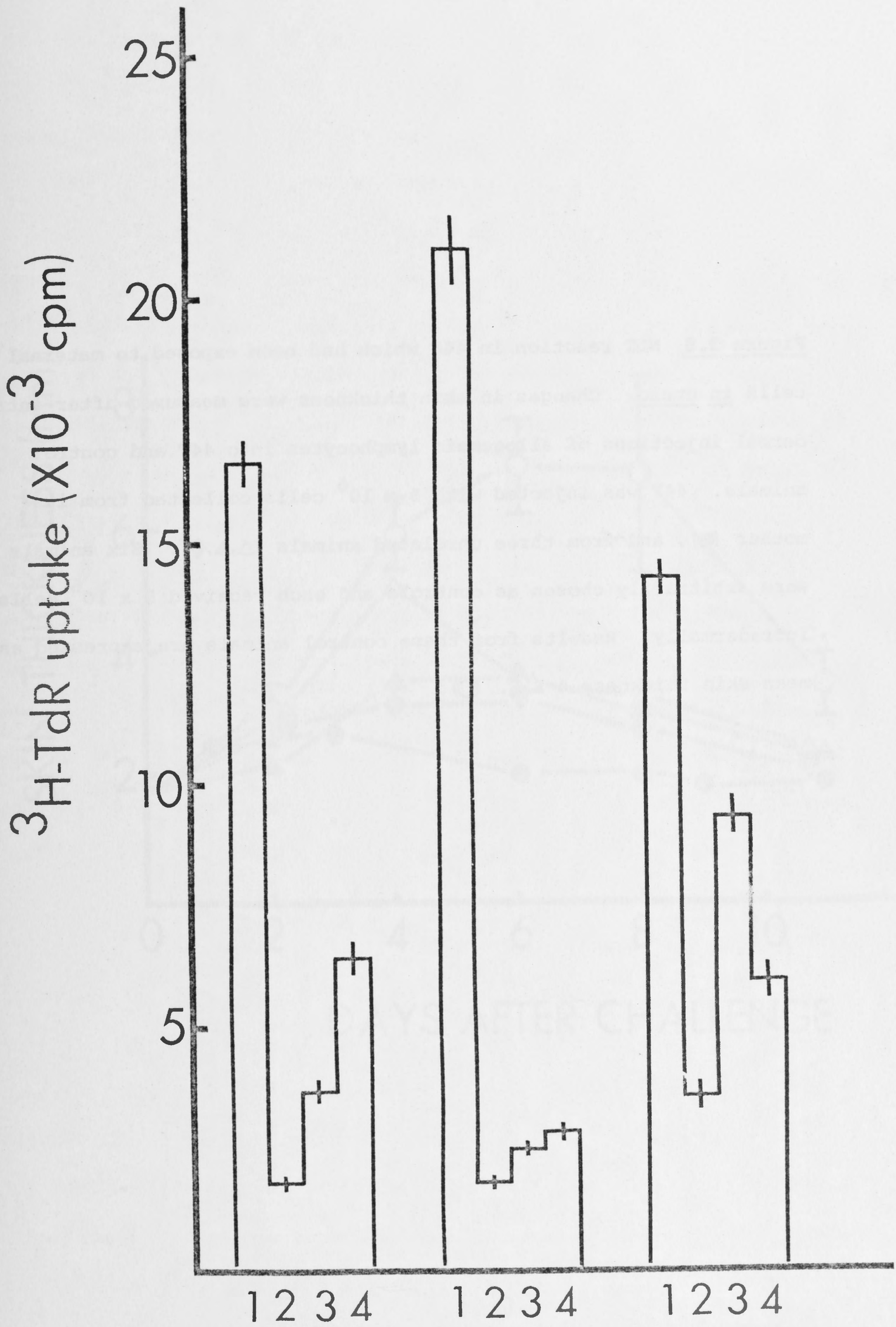
- maternal PBL in the first cluster of 4 columns,
- third-party PBL in the second and third clusters.

Each group of 4 columns represents the response:

1. in the absence of additional serum,
2. in the presence of 44F serum,

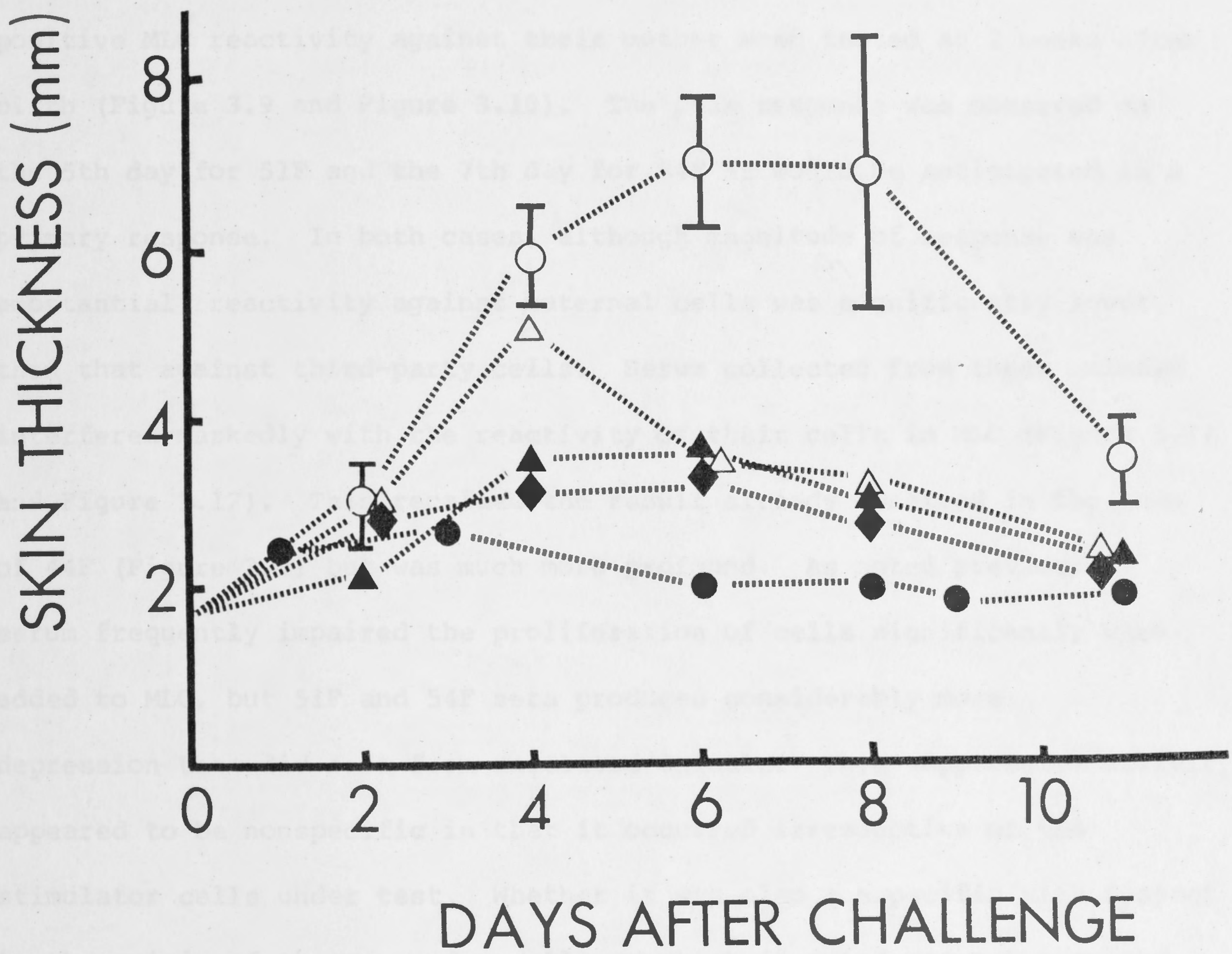
3 & 4. in the presence of sera from unrelated animals.

The final concentration of added serum was 10%. Results are expressed as mean cpm ± S.E. of triplicate cultures harvested on day 5.



^{3}H-TdR uptake (X10^3 cpm)
25
20
15
10
5
1 2 3 4
1 2 3 4
1 2 3 4

Figure 3.8 NLT reaction in 44F which had been exposed to maternal cells *in utero*. Changes in skin thickness were measured after intradermal injections of allogeneic lymphocytes into 44F and control animals. 44F was injected with 5×10^6 cells collected from its mother (●), and from three unrelated animals (△,▲,◆). Six animals were arbitrarily chosen as controls and each received 5×10^6 cells intradermally. Results from these control animals are expressed as mean skin thickness ± S.E. (○).


SKIN THICKNESS (mm)
8
6
4
2
0
2
4
6
8
10
DAYS AFTER CHALLENGE

cells whereas skin thickening was observed after injection of cells from 3 unrelated sheep. Responses of 44F to third-party cells were notable, as were the responses of 33F described earlier (Figure 3.5), for their aborted course. In all instances, responses appeared to be following a normal course for the first few days before abruptly ceasing.

Thirteen foetuses received suspensions of lymph node cells prepared, in each instance, from the prefemoral node of the mother (Table 3.1-C). From 2 x 10^7 to 10^8 viable cells were transferred. Only two out of the thirteen ewes produced lambs (51F and 54F). Both of these lambs showed positive MLC reactivity against their mother when tested at 2 weeks after birth (Figure 3.9 and Figure 3.10). The peak response was observed on the 5th day for 51F and the 7th day for 54F as would be anticipated in a primary response. In both cases, although magnitude of response was substantial, reactivity against maternal cells was significantly lower than that against third-party cells. Serum collected from these animals interfered markedly with the reactivity of their cells in MLC (Figure 3.11 and Figure 3.12). This recalled the result already observed in the case of 44F (Figure 3.7) but was much more profound. As noted previously, serum frequently impaired the proliferation of cells significantly when added to MLC, but 51F and 54F sera produced considerably more depression than did sera from unrelated animals. This suppressive activity appeared to be nonspecific in that it occurred irrespective of the stimulator cells under test. Whether it was also nonspecific with respect to the origin of the responder cells on which it acted was not examined experimentally. Both lambs rejected maternal grafts applied 6 months after birth at a normal rate. The NLT responses of these lambs to maternal and third-party cells are shown in Figure 3.13. 51F mounted a normal skin reaction to maternal cells while the reaction of 54F against maternal cells was slightly depressed.

The immunological responsiveness of the survivors are summarized in

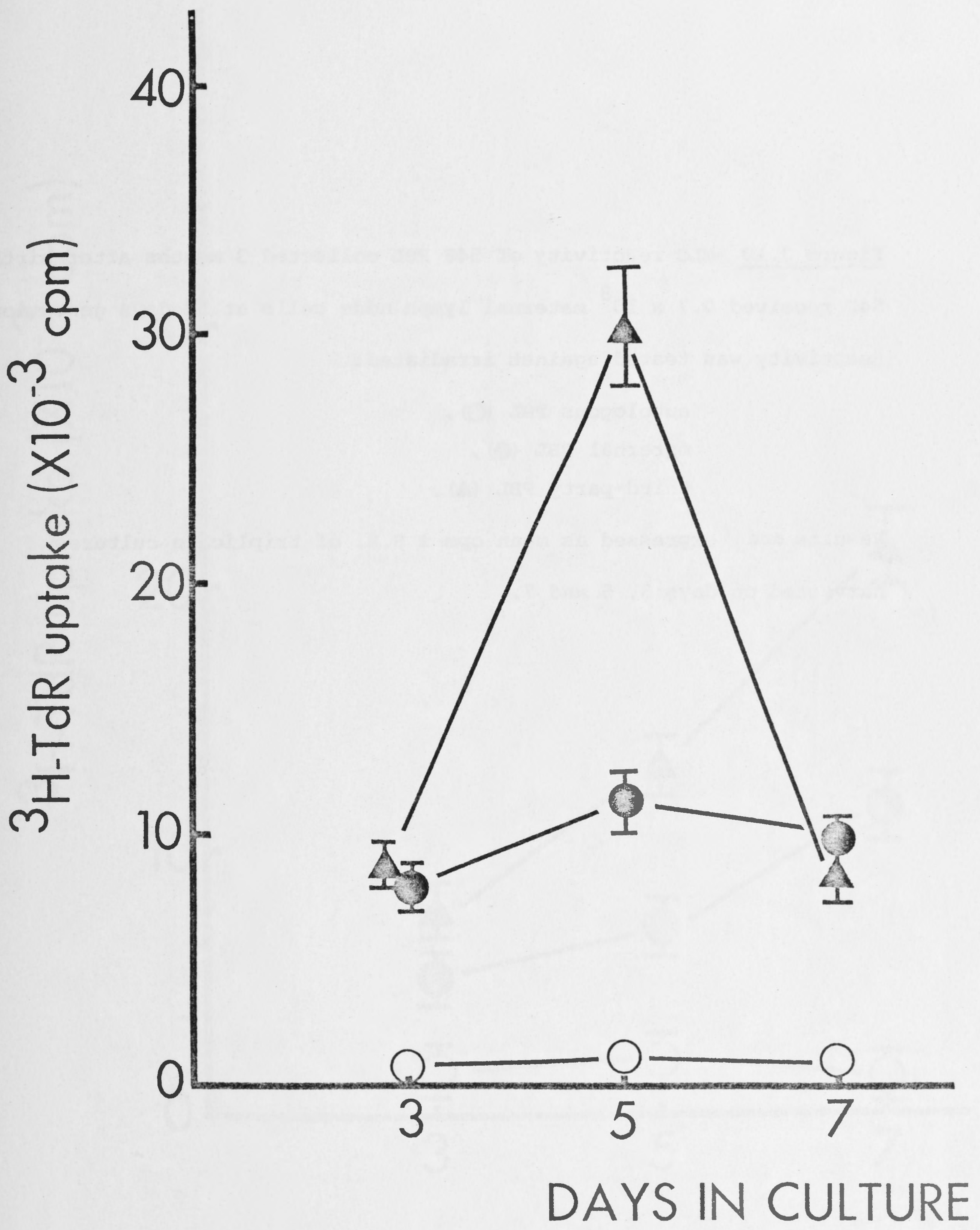
Figure 3.9 MLC reactivity of 51F PBL collected 2 weeks after birth. 51F received 10^8 maternal lymph node cells at 49 days of gestation. Reactivity was tested against irradiated:

autologous PBL (○),

maternal PBL (●),

third-party PBL (▲).

Results are expressed as mean cpm ± S.E. of triplicate cultures harvested on days 3, 5 and 7.



3H-TdR uptake (X10-3 cpm)
40
30
20
10
0
3
5
7
DAYS IN CULTURE

Figure 3.10 MLC reactivity of 54F PBL collected 3 months after birth. 54F received 0.7 x 10^8 maternal lymph node cells at 56 days gestation. Reactivity was tested against irradiated:

autologous PBL (○),
maternal PBL (●),
third-party PBL (▲).

Results are expressed as mean cpm ± S.E. of triplicate cultures harvested on days 3, 5 and 7.

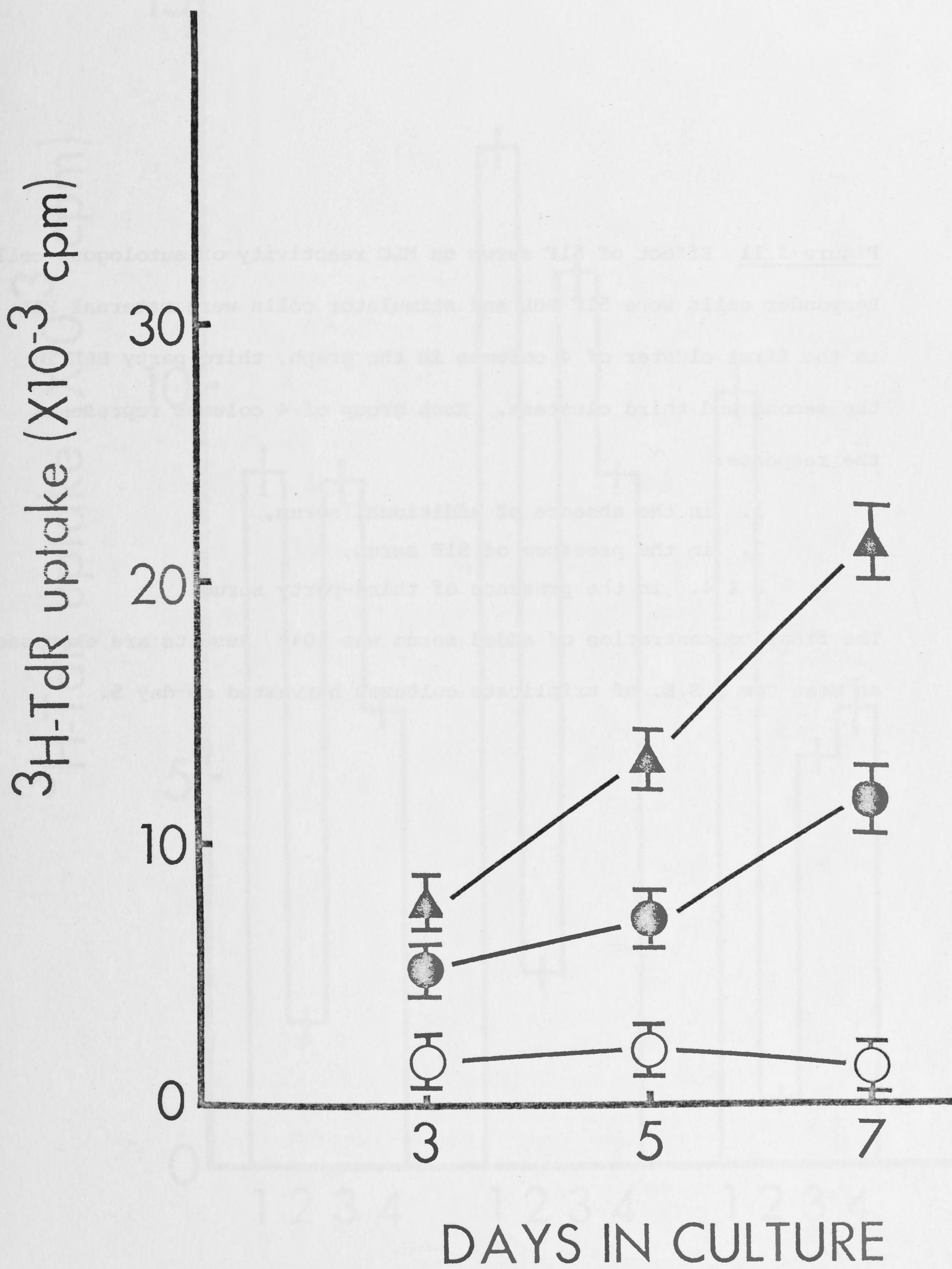
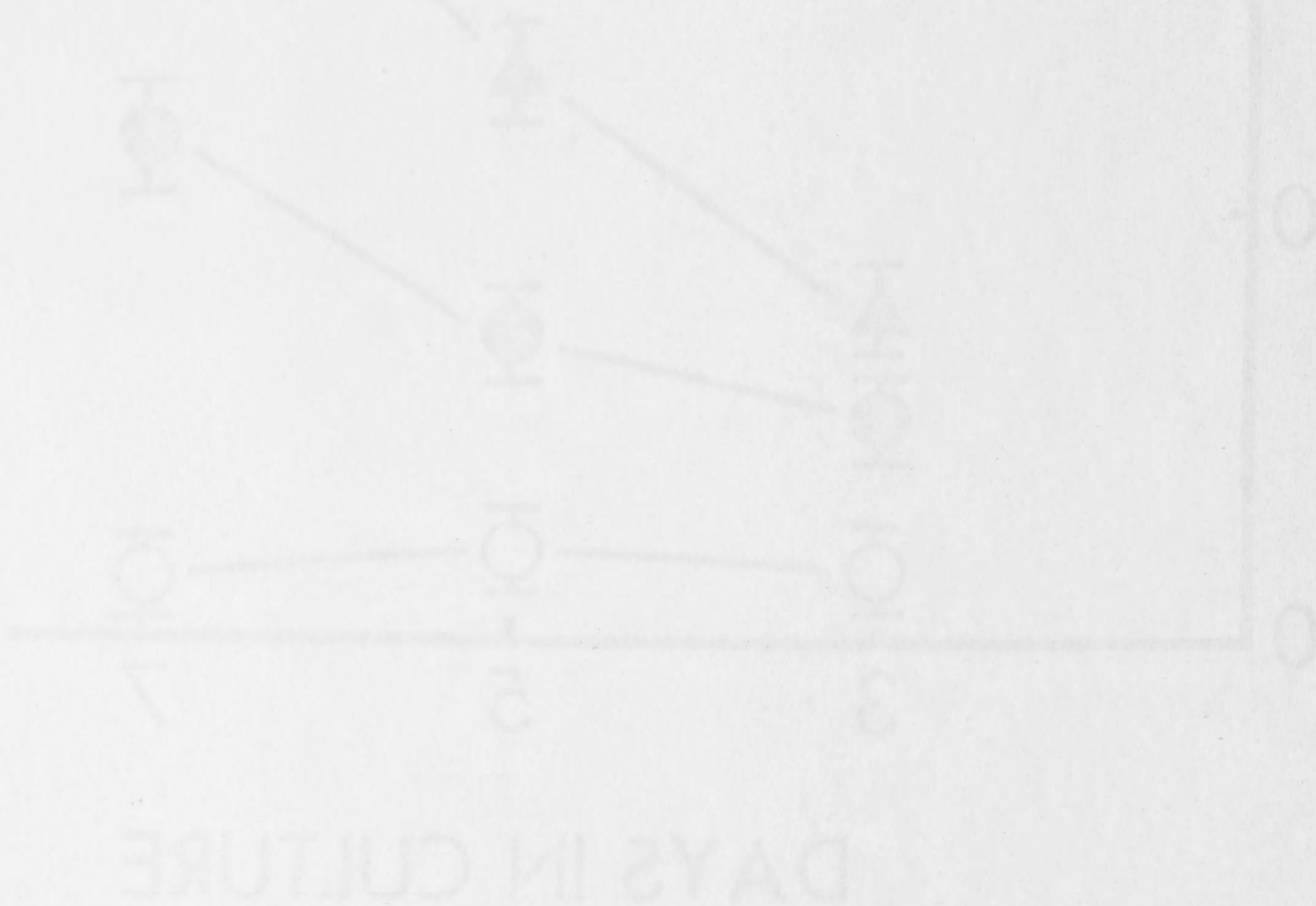
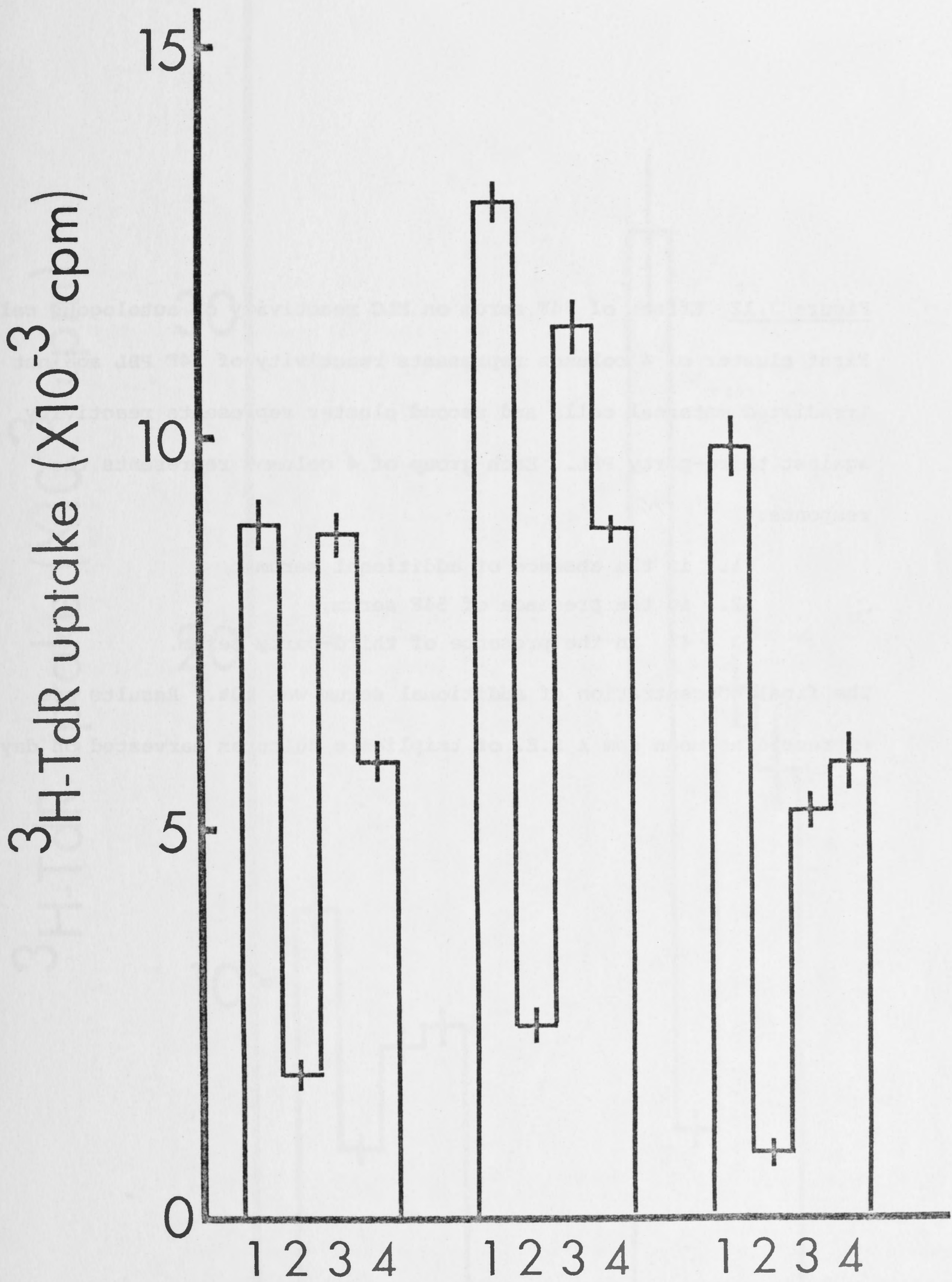

3H-TdR uptake (X10-3 cpm)
30
20
10
0
3
5
7
DAYS IN CULTURE

Figure 3.11 Effect of 51F serum on MLC reactivity of autologous cells. Responder cells were 51F PBL and stimulator cells were maternal PBL in the first cluster of 4 columns in the graph, third-party PBL in the second and third clusters. Each group of 4 columns represents the response:

1. in the absence of additional serum,
2. in the presence of 51F serum,
3 & 4. in the presence of third-party serum.

The final concentration of added serum was 10%. Results are expressed as mean cpm ± S.E. of triplicate cultures harvested on day 5.



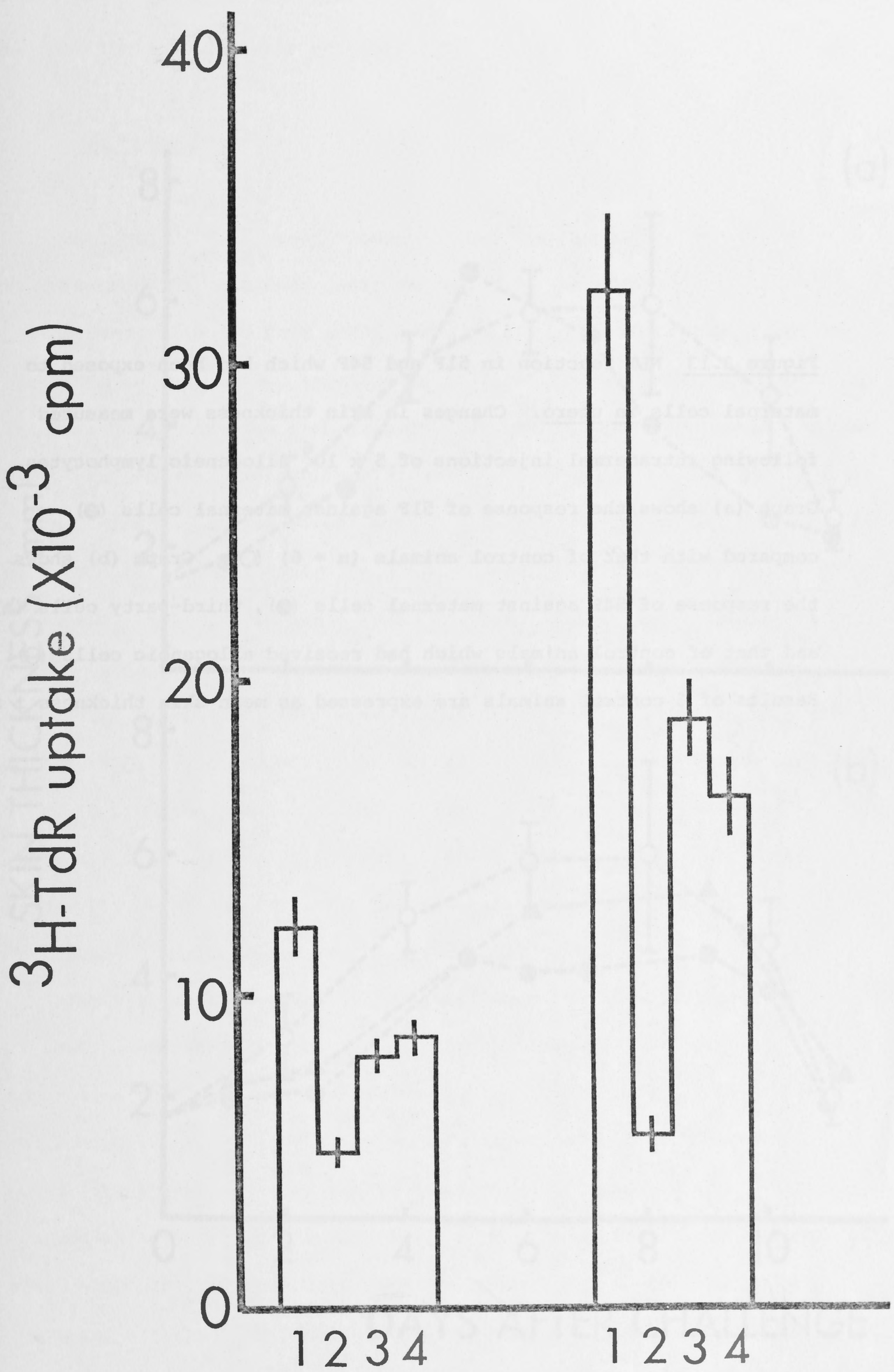


^{3}H-TdR uptake (X10^{-3} cpm)
15
10
5
0
1 2 3 4
1 2 3 4
1 2 3 4

Figure 3.12 Effect of 54F serum on MLC reactivity of autologous cells. First cluster of 4 columns represents reactivity of 54F PBL against irradiated maternal cells and second cluster represents reactivity against third-party PBL. Each group of 4 columns represents the response:

1. in the absence of additional serum,
2. in the presence of 54F serum,
3 & 4. in the presence of third-party serum.

The final concentration of additional serum was 10%. Results are expressed as mean cpm ± S.E. of triplicate cultures harvested on day 5.



3H-TdR uptake (X10-3 cpm)
40
30
20
10
0
1 2 3 4
1 2 3 4

Figure 3.13 NLT reaction in 51F and 54F which had been exposed to maternal cells *in utero*. Changes in skin thickness were measured following intradermal injections of 5×10^6 allogeneic lymphocytes. Graph (a) shows the response of 51F against maternal cells (●) compared with that of control animals (n = 6) (○). Graph (b) shows the response of 54F against maternal cells (●), third-party cells (▲) and that of control animals which had received allogeneic cells (○). Results of 6 control animals are expressed as mean skin thickness ± S.E.

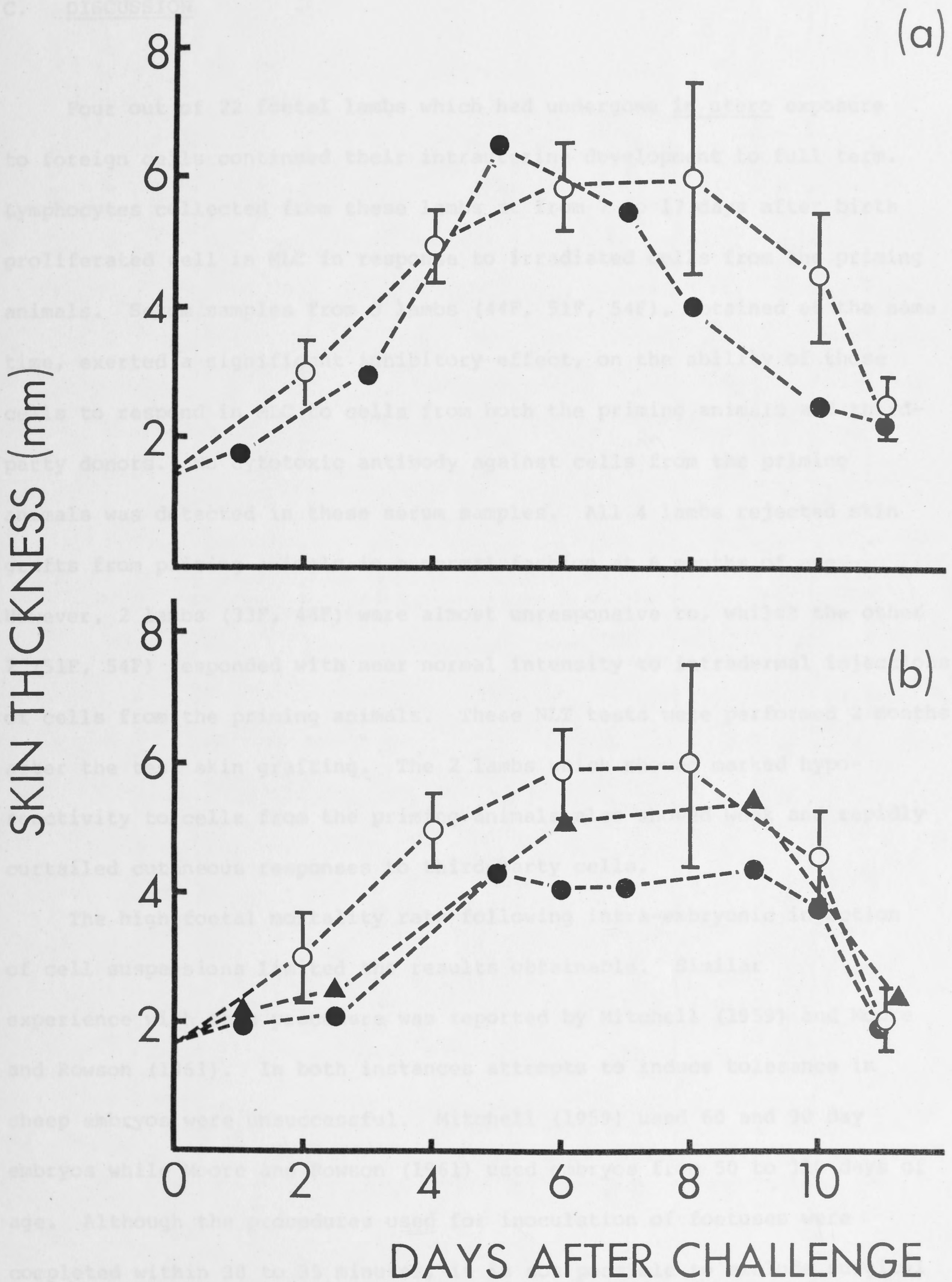

(a)
(b)
SKIN THICKNESS (mm)
8
6
4
2
0
2
4
6
8
10
DAYS AFTER CHALLENGE

Table 3.2. Tests for cytotoxic antibody in the serum of these lambs directed against the donor animals were negative.

C. DISCUSSION

Four out of 22 foetal lambs which had undergone *in utero* exposure to foreign cells continued their intrauterine development to full term. Lymphocytes collected from these lambs at from 7 to 17 days after birth proliferated well in MLC in response to irradiated cells from the priming animals. Serum samples from 3 lambs (44F, 51F, 54F), obtained at the same time, exerted a significant inhibitory effect, on the ability of those cells to respond in MLC to cells from both the priming animals and third-party donors. No cytotoxic antibody against cells from the priming animals was detected in these serum samples. All 4 lambs rejected skin grafts from priming animals in a normal fashion at 6 months of age. However, 2 lambs (33F, 44F) were almost unresponsive to, whilst the other 2 (51F, 54F) responded with near normal intensity to intradermal injections of cells from the priming animals. These NLT tests were performed 2 months after the test skin grafting. The 2 lambs which showed marked hypo-reactivity to cells from the priming animals also showed weak and rapidly curtailed cutaneous responses to third-party cells.

The high foetal mortality rate following intra-embryonic injection of cell suspensions limited the results obtainable. Similar experience with this procedure was reported by Mitchell (1959) and Moore and Rowson (1961). In both instances attempts to induce tolerance in sheep embryos were unsuccessful. Mitchell (1959) used 60 and 90 day embryos while Moore and Rowson (1961) used embryos from 50 to 110 days of age. Although the procedures used for inoculation of foetuses were completed within 30 to 35 minutes, it is not possible to exclude surgical trauma or stress as the cause of the foetal wastage. However, it is likely

Table 3.2 Summary of immunological responsiveness of foetuses which had been exposed to foreign cells *in utero*.

Foetus	Stage of gestation at challenge (days)	Source transfer of cells	No. of cells transferred ($x10^{-8}$)	MLC reactivity	Serum suppressive activity	Cytotoxic antibody in serum	NLT reactivity	Survival time of skingraft from the priming animal (days)
33F	73	allo BM[1)]	2.0×10^8	positive	None detectable	negative	markedly decreased non-specific (?)	9 days
44F	59	mat BM[2)]	1.8×10^8	positive	(+) Non-specific	negative	markedly decreased non-specific (?)	9 days
51F	49	mat LN[3)]	1.0×10^8	positive	(+) Non-specific	negative	Normal	9 days
54F	56	mat LN	0.7×10^8	positive	(+) Non-specific	negative	Slightly decreased specificity (?)	9 days

1) allogeneic bone marrow cells

2) maternal bone marrow cells

3) maternal lymph node cells

that aggression by the transferred lymphocytes was responsible for much of the high foetal mortality. It has been shown that allogeneic immunocompetent cells express aggression in the form of a fatal GVHR in newborn mice (Billingham and Brent 1957) and newly hatched chicks (Cock and Simonsen 1958). In the current experiments, third-party lymphocytes would be expected to be fully competent and the reactivity of maternal cells against foetal determinants has been documented in Chapter 1.

The cell doses employed (2.0×10^8 bone marrow cells to a 73 day old embryo (33F), 1.8×10^8 bone marrow cells to a 59 day old embryo (44F), 10^8 lymph node cells to a 49 day old embryo (51F) and 0.8×10^8 lymph node cells to a 56 day old embryo (54F)) were arbitrarily chosen because of the impracticability of using large numbers of embryos to determine the appropriate dose. The doses used for the 3 younger foetuses (44F, 51F 54F) were more than 3 times higher per unit body weight than those used by Binns (1967) (6×10^8 nucleated lymph node cells/kg and 13.5×10^8 nucleated bone marrow cells/kg of body weight) in pigs and about 20 times higher than those used by Mitchell (1959) (0.2 to 0.4×10^8 spleen cells intramuscularly into 90 day old sheep embryos). The latter worker also failed to induce tolerance of kidney homografts by inoculation of foetal lambs. The body weight of foetal lambs was estimated as described by Stephenson (1959) and Stephenson and Lambourne (1969). The inoculated cells were freshly prepared and there is no doubt that they were accurately injected into the foetuses because the inoculum was observed to disperse in the peritoneal cavity during injection.

Silverstein, Prendergast and Kraner (1964) reported that skin grafts orthotopically applied to foetal lambs about the 65th day of gestation were accepted and retained for up to 21 days without signs of rejection and that all lambs older than 77 days gestation effectively rejected skin

grafts within 7 to 10 days, the implication being that the foetal lamb of 65 days was still in an 'adaptive' stage. However, in the present experiments, although 3 foetal lambs (44F, 51F, 54F) received large numbers of immunocompetent maternal cells before 65 days gestation, lymphocytes collected from these lambs after birth retained the capacity to react against maternal cells and, furthermore, these lambs rejected maternal skin grafts, applied 6 months after birth, with normal speed. This suggests that the foetus of 50 to 60 days of age possesses some ability to protect itself against immunological attack by maternal or allogeneic cells. The demonstration that neonatal F_1 hybrid strain rats which are in most other respects immuno-incompetent are able to respond to and subsequently inactivate parental strain cells (McCullagh 1977) may be relevant.

Although cytotoxic activity was absent, suppressive activity was detected in serum from 3 lambs (44F, 51F, 54F). Although its significance is not clear, it is notable that serum suppressive activity was detected in 3 lambs (44F, 51F, 54F) which had had *in utero* exposure to *maternal cells* but not in a lamb (33F) which was exposed to unrelated cells *in utero*. All 3 lambs were exposed to maternal cells before 60 days *in utero*, whereas the remaining one was exposed to allogeneic cells at 73 days gestation (according to Silverstein, Prendergast and Kraner (1964) the 'adaptive' period ends between 65 and 77 days gestation). Serum generally produced a marked depression of the reactivity of autologous cells when added to MLC, but serum from these 3 lambs produced significantly more depression than did third-party serum samples. This depression was observed with both maternal, and third-party cells as stimulators. The specificity of the suppressive effect with regard to the responder cells under test was not investigated.

Histocompatibility between 2 arbitrarily chosen animals may well be a more common finding than expected in sheep given the fact that it is a

common practice to use few rams for breeding from a large number of ewes. Unfortunately, as the extent of genetic proximity between maternal and third-party animals was not known in the present study, caution is necessary in interpretation of the specificity of immune response in sheep.

It remains speculative whether the suppressive activity detected in lamb serum was derived from recipient cells or perhaps from persisting donor (maternal) cells. Nevertheless, it is conceivable that any donor cells which persisted in the recipient would react against recognition structures on foetal cells the specificity of which was directed against alloantigens of donor cells. Subsequently, the donor cells might mount an anti-idiotypic response to counteract the foetal response. Anti-idiotypic responses against alloreactive cells have been demonstrated in some studies (McKearn 1973, Binz and Wigzell 1975, Bellgrau and Wilson 1978) and inferred in others (McCullagh 1973, 1977).

The depressed NLT reactivity observed in 2 of the lambs (33F, 44F) requires comment. The response of both lambs against donor cells was almost nil and curtailed responses were also observed against third-party cells. The NLT reaction is a cutaneous inflammatory response of delayed onset which can be elicited by intradermal challenge with allogeneic immunocompetent cells and has been described as a local GVHR (Brent and Medawar 1962, Ramseier and Billingham 1966). Decreased or negative NLT reactivity has been observed in several experimental situations;

(1) in lambs thymectomized at around 70 days of age <u>in utero</u> (Cole and Morris 1973),

(2) in normal animals challenged with lymphocytes from tolerant donors (Terasaki 1959, Ford 1967),

(3) in animals presensitized against antigens of the donor animals (Terasaki 1959, Amos et al. 1965, Jones and Lafferty 1965, Emery 1978)(the reaction was often curtailed in these animals, presumably because of inactivation of transferred cells by the immune host).

In the present study, test skin grafting was performed 2 months prior to the NLT test as it was feared that intradermal injection of allogeneic cells into the lambs might affect the outcome of the test grafts. Although the extent of interference with the NLT reaction by the previous test skin grafting is not known and very few lambs were available, it is interesting that two lambs, which had bone marrow rather than lymph node cells as foetuses, developed poor cutaneous responses to allogeneic cells particularly to the priming cells. The significance of the curtailed and abortive responses observed against third-party cells is not clear as the extent of genetic proximity between donor and third-party animals was not investigated in this study. The similarity to the behaviour of _in utero_ thymectomized lambs in dissociation between NLT reactivity and graft rejection (Cole and Morris 1973) is also notable.

CHAPTER 4

THE IN VITRO REACTIVITY OF FOETAL LYMPHOCYTES

A. INTRODUCTION

While the examination of cell behaviour in vitro can be potentially misleading, if not correlated with performance in the intact animal, assessment of lymphocyte function in culture before examination of the immune response of the whole foetus offered a means of simplifying interpretation of the latter. As a number of in vitro assays were to be used in later experiments the aim of which was to examine the effect on immunological homeostasis between mother and foetus of the deliberate transfer of maternal cells to the foetus, it was appropriate to assess the performance of lymphocytes from unchallenged foetuses in these assays first. The experiments described in this chapter relate to the capacity of foetal lymphocytes to proliferate in response to immunologically specific and nonspecific stimuli and to act as a stimulus for the proliferation of allogeneic cells. The effect of foetal plasma on foetal lymphocyte reactivity in vitro has also been investigated.

B. RESULTS

4.1 REACTIVITY OF FOETAL THORACIC DUCT LYMPHOCYTES

The MLC was used to assess responsiveness of foetal cells. This assay was performed to examine both the specific response of foetal cells to maternal or paternal cells and their response to cells from unrelated donors. While the latter situation is conventionally referred to as 'nonspecific', it is clear that nonspecificity of any third-party can not be guaranteed as cell donors could not be typed for histocompatibility. Lymphocytes obtained from the thoracic duct of 10 foetuses (from 110 to 127 days gestation) were tested for MLC reactivity against parental and third-party PBL and for their reactivity to Con A (Table 4.1). Preliminary results

Table 4.1 Reactivity of foetal thoracic duct lymphocytes in mixed lymphocyte culture and in response to Concanavalin A.

Foetus	Stage of gestation (days)	Tritiated thymidine uptake (mean c.p.m. ± S.E. ($x10^{-3}$)) against a) autologous cells	b) maternal cells	c) paternal cells	d) third-party cells	e) Concanavalin A
29F	110	0.9 ± 0.1	65.5 ± 1.2	129.0 ± 1.2	99.5 ± 1.7	134.3 ± 12.2
23F	113	0.6 ± 0.1	18.8 ± 0.4	n.d.[1)]	50.6 ± 3.1	12.8 ± 1.8
50F	116	0.2 ± 0.1	60.4 ± 3.5	56.0 ± 0.5	67.5 ± 1.2	107.7 ± 15.3
84F	117	0.2 ± 0.1	19.8 ± 0.6	24.6 ± 0.2	n.d.	49.7 ± 1.8
27F	120	0.3 ± 0.1	30.3 ± 2.1	n.d.	30.0 ± 0.8	23.5 ± 1.6
40F	121	3.4 ± 0.1	45.7 ± 2.1	72.5 ± 3.1	73.5 ± 5.2	181.1 ± 3.1
28F	121	0.4 ± 0.1	37.5 ± 0.2	n.d.	44.1 ± 0.9	28.0 ± 1.8
36F	125	0.5 ± 0.1	109.8 ± 5.6	n.d.	108.0 ± 4.9	n.d.
31F	127	9.7 ± 0.3	42.1 ± 1.5	n.d.	55.1 ± 2.5	n.d.
Adult	2 years old	0.3 ± 0.1	34.3 ± 2.0	38.2 ± 3.2	29.9 ± 2.3	68.2 ± 3.0

Stimulator cells were foetal thoracic duct lymphocytes in (a) and peripheral blood lymphocytes from donors of the indicated type in (b), (c) and (d). When adult thoracic duct lymphocytes were responders, all stimulator cells were third-party cells. Cultures were harvested on the fifth day in the case of (a)-(d) and on the third day in the case of (e). In some instances additional cultures of types (a)-(d) were harvested on the third and seventh days. The results obtained in this way were comparable with those presented in this table.

1) n.d. not determined.

showed that foetal lymphocyte reactivity to both maternal and third party PBL (Figure 4.1-(a),(b)) was comparable to that of adult cells. (Figure 4.1-(c)) in tempo and magnitude. The optimal dose of Con A for stimulation of foetal thoracic duct lymphocytes was the same as that for adult thoracic duct lymphocytes (10 to 20 μg/ml; Figure 4.2). As shown in Table 4.1, in 5 out of 9 instances, the MLC reactivity of foetal thoracic duct lymphocytes against maternal PBL was compatible in magnitude with their reactivity against cells from unrelated third-party donors. Genetic proximity might be expected to result in some instances in which even the mature offspring might respond poorly against maternal cells and this proximity may well account for those instances in which the anti-maternal reactivity of foetal cells was comparatively reduced. The MLC reactivity of foetal cells against unrelated PBL was also comparable in magnitude with that of adult cells against unrelated cells. These results suggest that foetal lymphocyte alloreactivity as expressed *in vitro* resembles that of cells from adult animals. Con A reactivity showed a wide variation with some foetal cells responding better than the adult cells used in this experiment.

4.2 STIMULATORY CAPACITY OF FOETAL PBL

When maternal reactivity against foetal determinants had been investigated, as reported in Chapter 1, foetal thoracic duct lymphocytes were used as stimulators of maternal PBL because a reliable technique for separating lymphocyte populations from whole foetal blood was not available. Although foetal thoracic duct lymphocytes were poor stimulators of PBL from non-pregnant ewes, the efficacy with which they stimulated cells from many ewes in late pregnancy implied that under appropriate conditions foetal cells are effective stimulators. Testing this inference was possible when the technique of separating lymphocytes

Figure 4.1 MLC kinetics of foetal and adult thoracic duct lymphocytes. Foetal thoracic duct cells were cultured with irradiated maternal PBL (●) and irradiated third-party PBL (○) (A and B), and adult cells were cultured with irradiated PBL from 2 unrelated animals (▲,■) (C). Results are expressed as mean cpm ± S.E. of triplicate cultures harvested on days 3, 5 and 7.

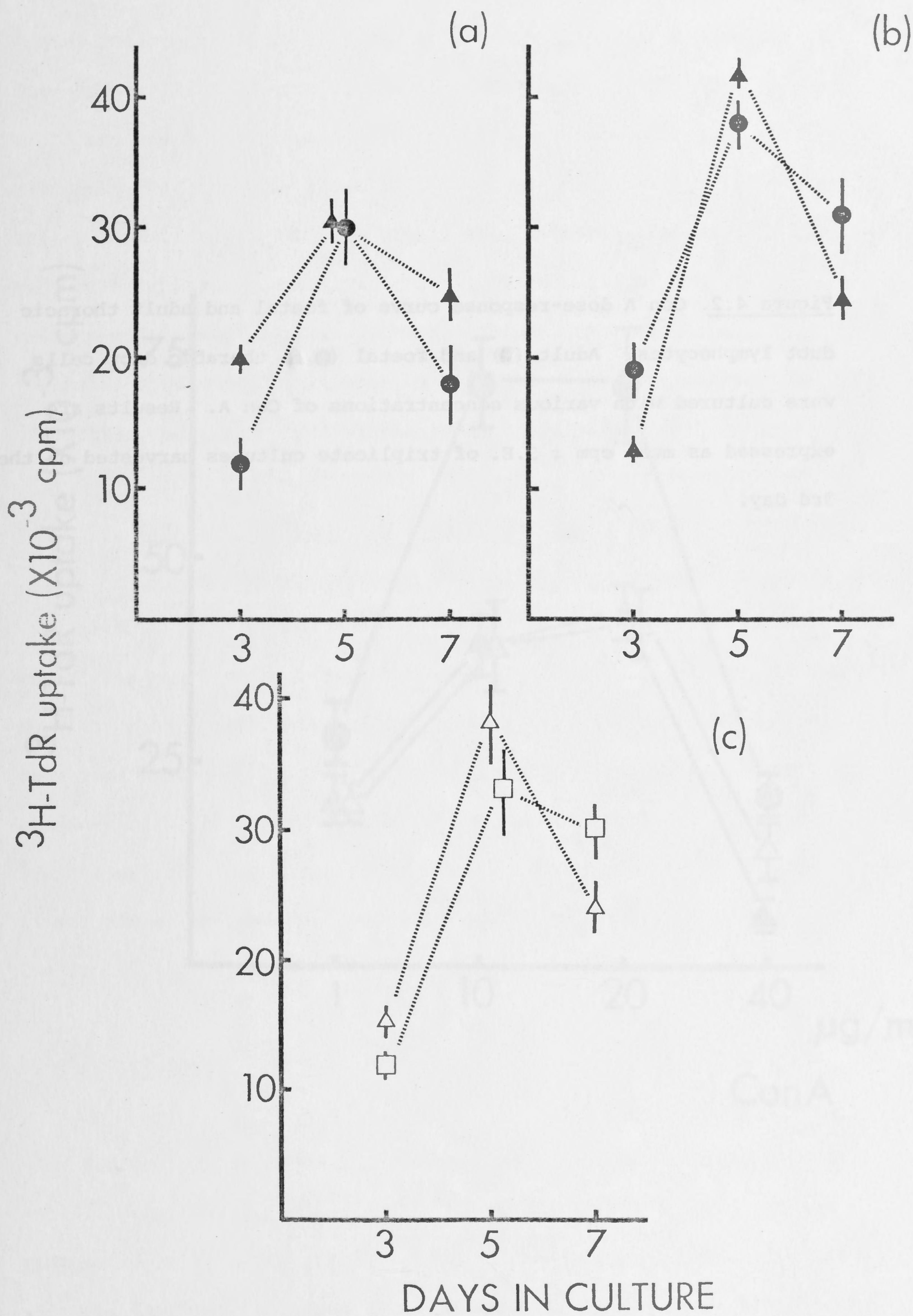
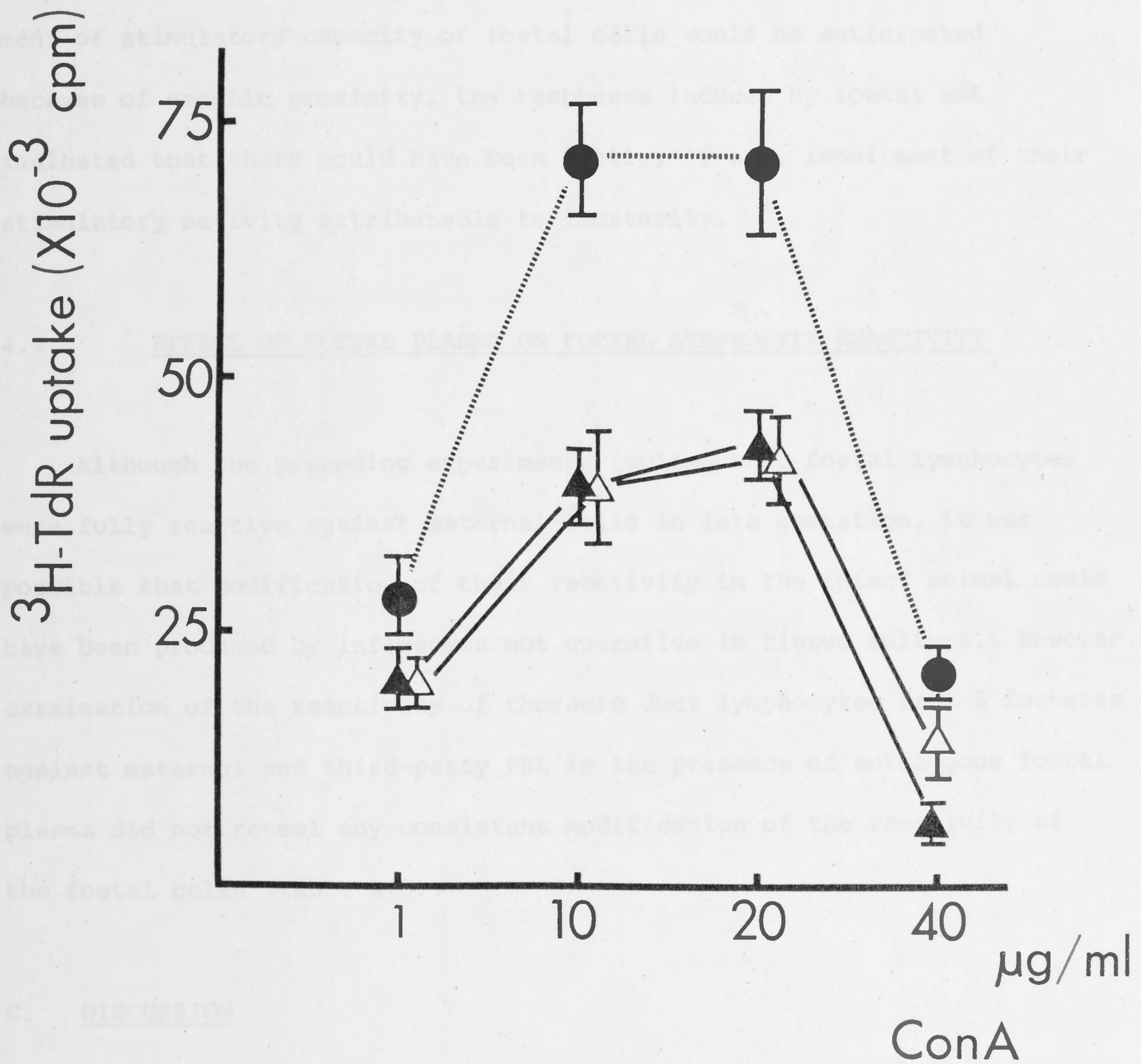

(a)
(b)
(c)
3H-TdR uptake (X10-3 cpm)
40
30
20
10
3
5
7
DAYS IN CULTURE

Figure 4.2 Con A dose-response curve of foetal and adult thoracic duct lymphocytes. Adult (●) and foetal (●,△) thoracic duct cells were cultured with various concentrations of Con A. Results are expressed as mean cpm ± S.E. of triplicate cultures harvested on the 3rd day.



^{3}H-TdR uptake (X10^{-3} cpm)
75
50
25
1
10
20
40
μg/ml
ConA

from foetal blood by means of Ficoll-Isopaque isopycnic centrifugation was supplemented by warm Tris-NH_4Cl lysis of contaminating red cells. Foetal PBL separated in this manner from the blood of 6 foetuses of from 113 to 125 days were tested as stimulators in MLC. The responder cells in each instance were cryopreserved maternal PBL collected before pregnancy. As shown in Table 4.2, maternal cells usually responded better to third-party than to foetal PBL. Nevertheless, as some impairment of stimulatory capacity of foetal cells would be anticipated because of genetic proximity, the responses induced by foetal PBL indicated that there could have been little, if any, impairment of their stimulatory activity attributable to immaturity.

4.3 EFFECT OF FOETAL PLASMA ON FOETAL LYMPHOCYTE REACTIVITY

Although the preceding experiments implied that foetal lymphocytes were fully reactive against maternal cells in late gestation, it was possible that modification of their reactivity in the intact animal could have been produced by influences not operative in tissue culture. However, examination of the reactivity of thoracic duct lymphocytes from 5 foetuses against maternal and third-party PBL in the presence of autologous foetal plasma did not reveal any consistent modification of the reactivity of the foetal cells (Table 4.3).

C. DISCUSSION

Responsiveness of foetal lymphocytes to agents designated as 'T cell mitogens' (PHA and Con A) has been extensively studied. PHA responsive cells have been identified in human foetal thymus from 10 (Stites, Carr and Fudenberg 1974), or 12 weeks gestation (Papiernik 1970, Haywood and Soothill 1972, Haywood 1973), in pig foetal thymus from

Table 4.2 Comparative stimulating activity of foetal and third-party PBL.

Foetus	Gestational age of foetus when PBL was collected (days)	Tritiated thymidine uptake (mean c.p.m. ± S.E. ($x10^{-3}$)) of maternal cells against		
		autologous PBL	foetal PBL	third-party PBL
23F	113	2.9 ± 0.1	21.00 ± 2.0	39.5 ± 3.7
35F	119	0.6 ± 0.1	61.9 ± 2.5	35.9 ± 0.7
31F	123	1.2 ± 0.1	22.1 ± 1.1	31.5 ± 1.0
22F	125	11.0 ± 1.3	33.6 ± 0.9	50.1 ± 1.3
24F	125	0.5 ± 0.1	46.8 ± 1.8	48.9 ± 1.5
26F	125	2.6 ± 0.1	78.0 ± 1.5	90.3 ± 2.4

1) Responder cells were cryopreserved maternal PBL collected before pregnancy.

2) Stimulator cells were either foetal PBL collected at the designated time of gestation or third-party PBL both of which had been cryopreserved.

3) Cultures were harvested on the 5th day.

Table 4.3 Influence of foetal plasma on response of foetal lymphocytes in mixed lymphocyte culture.

Foetal cell and plasma donor	Stage of gestation (days)	Medium supplement	Tritiated thymidine uptake (mean c.p.m. ± S.E. ($x10^{-3}$)) against a) autologous TDL	b) maternal PBL	c) third party PBL
		none		not measured	
29F	110	FCS	1.2 ± 0.1	16.5 ± 1.1	24.5 ± 1.5
		FLP	1.1 ± 0.1	13.5 ± 0.6	21.6 ± 1.8
		none		not measured	
33F	113	FCS	0.2 ± 0.1	10.3 ± 0.9	34.5 ± 1.2
		FLP	0.6 ± 0.1	14.7 ± 1.9	64.2 ± 0.2
		none	0.4 ± 0.04	67.1 ± 2.7	not measured
35F	119	FCS	0.4 ± 0.04	32.9 ± 1.2	not measured
		FLP	0.5 ± 0.07	57.6 ± 0.5	not measured
		none	0.5 ± 0.04	48.6 ± 1.9	95.3 ± 1.3
40F	121	FCS	0.5 ± 0.05	31.6 ± 1.5	64.1 ± 2.7
		FLP	0.4 ± 0.02	40.9 ± 1.6	37.3 ± 1.2
		none	0.6 ± 0.1	22.3 ± 1.5	47.8 ± 2.6
26F	134	FCS	0.6 ± 0.1	13.9 ± 0.1	42.6 ± 3.0
		FLP	0.7 ± 0.1	13.1 ± 1.1	48.7 ± 1.9

Foetal thoracic duct lymphocytes, which were used as responder cells, and jugular vein plasma were collected within one or two days of each other, the indicated time being that applicable to plasma collection. Stimulator cells were either autologous foetal thoracic duct lymphocytes (TDL), maternal peripheral blood lymphocytes (PBL), collected in the post-partum period, or third-party peripheral blood lymphocytes. The culture medium was supplemented as indicated with 10% of either autologous foetal lamb plasma (FLP) or foetal calf serum (FCS), each of which contained sufficient heparin to result in a final medium concentration of 2.5 units/ml. All cultures were performed in triplicate and harvested on the fifth day.

72 days gestation (Rodey et al. 1972), in foetal sheep thymus from 68 days gestation (Leino 1978), in foetal guinea pig spleen from 36 days gestation (Leiper and Solomon 1977); and in foetal mouse thymus from 18 days gestation (Mosier 1974). However, mitogen reactivity should not automatically be accepted as indicative of functional capacity. Thus, mouse thymocytes (Stobo and Paul 1972), MLC activated T cells (Andersson and Häyry 1973) and lymphocytes from patients with congenital thymic dysplasia (Meuwissen, Bach and Good 1968) all respond well to allogeneic cells but poorly to PHA. The reactivity of foetal cells has also been used to compare their immunocompetence with that of adult cells. A lower (Jones 1969, Ayoub and Kasakura 1971) or similar (Davis and Galant 1975) response to PHA by cord blood cells compared to adult cells has been reported. The disparate observations of these groups were possibly due to the different dosage of PHA employed and to variation in other culture conditions. _In vitro_ proliferation assays have been shown to be subject to many variables (Wilson 1966, Bloom and David 1976). Careful studies by Carr, Stites and Fudenberg (1972) showed that human cord lymphocytes were more responsive to low PHA doses than adult cells but that the reactivity of both populations was comparable at intermediate and high dose.

The present investigation indicated that the maximum response of foetal sheep lymphocytes was evoked by a concentration of Con A that was also the most efficacious for stimulation of adult cells. Although foetal thoracic duct cells showed a wide range of reactivity, the response of some populations was similar in magnitude to the response of adult cells. Foetal thoracic duct cells also appeared to react with similar intensity against both maternal cells and cells from unrelated donors. The MLC has been used as a tool for investigation of ontogeny of alloreactivity. For example, MLC reactivity has been detected in human foetal liver cells from as early as 7½ weeks gestation, that is well

before appearance of the thymus (Stites, Carr and Fudenberg 1974). Asantila and Toivanen (1976) has shown in foetal lamb that allo-reactivity develops in liver from 41 days and in thymus from 47 days gestation.

The stimulatory capacity of foetal cells has been less extensively studied than has their capacity to respond. It has been shown that human cord blood lymphocytes satisfactorily stimulated maternal cells in MLC but to a lesser extent than did unrelated cells (Carr, Stites and Fudenberg 1974) and this was ascribed to genetic similarities between foetus and mother. The current study, in accord with the results obtained by Carr, Stites and Fudenberg (1974) shows that foetal lamb PBL collected in late gestation are able to stimulate maternal cells significantly although their stimulatory capacity may have been slightly inferior to that of adult cells. Whether this was due to the one-haplotype identity or not cannot be determined from this study.

The instrinsic reactivity of lymphocytes *in vivo* has been shown to be subject to a variety of humoral factors (Stites et al. 1979) such as alpha-fetoprotein (AFP) which has been claimed to be immunosuppressive (Murgita and Tomasi 1975a, 1975b, Yachnin 1976, Gupta and Good 1977, Auer and Kress 1977). While it is an open question whether substances such as AFP are immunosuppressive in some species, the current observation that foetal lamb plasma did not affect the responsiveness of foetal thoracic duct lymphocytes *in vitro* suggests that significant modification of reactivity of these cells is unlikely to take place in the foetal sheep.

CHAPTER 5

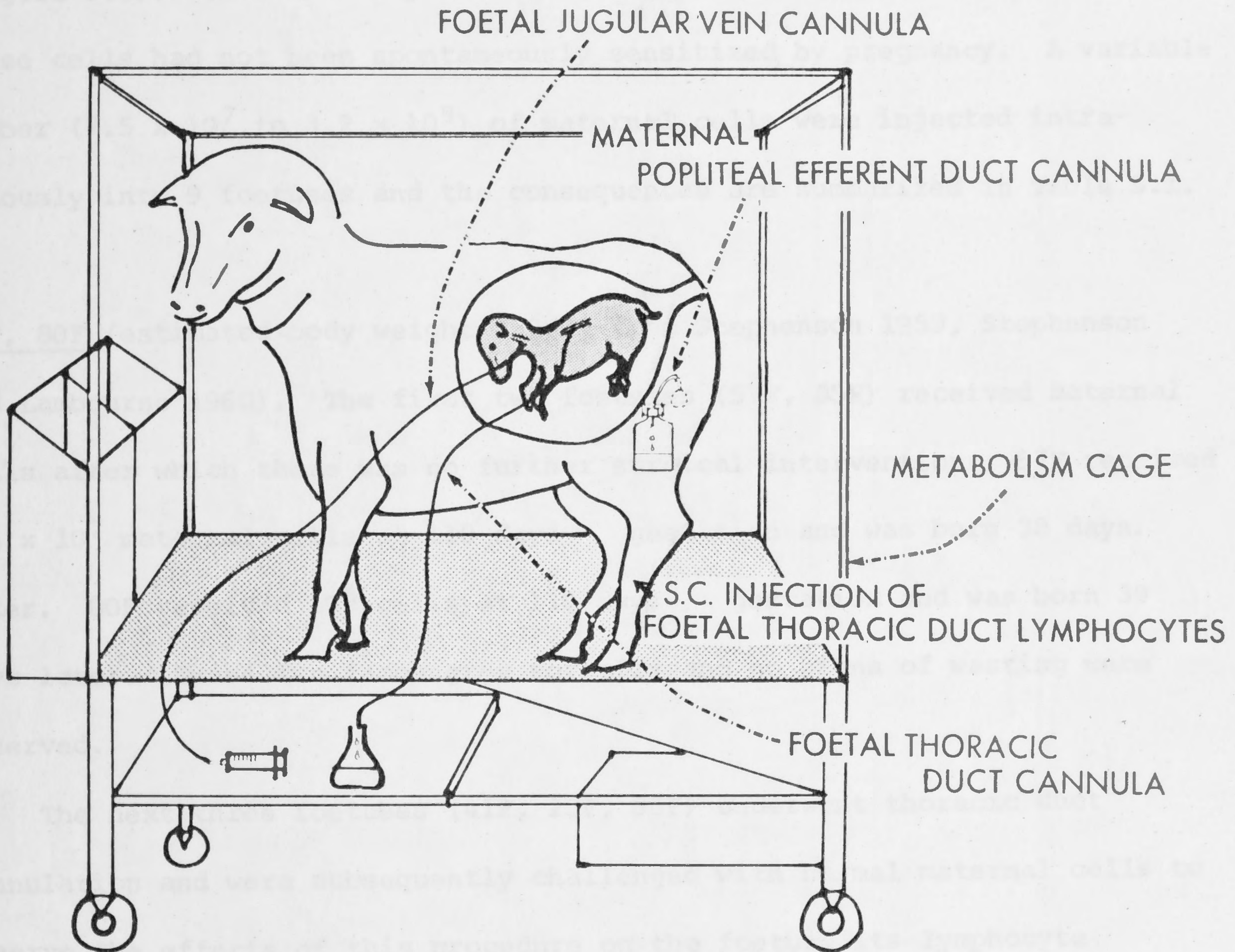
THE EFFECTS OF ARTIFICIAL TRANSFER OF MATERNAL LYMPHOCYTES TO THE FOETUS IN LATE GESTATION

A. INTRODUCTION

The experiments in Chapter 3 examined the response of foetuses between 49 and 73 days gestation to the transfer of lymphoid cells from maternal and third-party donors. While clear indications of some immunological modifications were apparent in the minority of foetuses surviving to term, the diversity of responses in different assays precluded any simple categorization of these animals as tolerant or immune. It was shown in Chapter 4 that the reactivity of cells collected from older foetuses (from 110 to 134 days) was indistinguishable from that of adult cells *in vitro* and that foetal plasma did not inhibit this reactivity to a significant extent.

This chapter is concerned with the *in vivo* response of older foetuses (from 110 to 150 days) to immunocompetent maternal cells. The experiments were designed to permit continuous monitoring of the responses of the lambs against maternal lymphocytes, introduced via a jugular vein cannula, as reflected in the reactivity of the recipient's thoracic duct lymphocytes. Before foetal jugular and thoracic duct cannulation, the popliteal efferent lymphatic duct of the pregnant ewes had been cannulated in order to provide a large number of maternal lymphocytes for injection into the foetus. On those occasions when specifically immunized lymphocytes were required, the popliteal node was challenged with foetal thoracic duct or paternal efferent lymphatic cells (Figure 5.1). As a major objective of this study was to test the potential of any mechanisms responsible for maintaining foetal integrity in the face of possible immunological attack by the mother, numbers of maternal cells far in excess of those ever likely to cross the placenta spontaneously were injected into foetus.

Figure 5.1 Diagram of the experimental model. A plastic catheter was inserted into a maternal lymphatic duct efferent to the popliteal lymph node to collect large numbers of maternal lymphocytes. The foetus *in utero* carries two cannulae, namely a jugular vein cannula through which maternal cells can be injected into the foetus, and a thoracic duct cannula from which lymph samples can be collected over periods of weeks after challenge.


FOETAL JUGULAR VEIN CANNULA
MATERNAL
POPLITEAL EFFERENT DUCT CANNULA
METABOLISM CAGE
S.C. INJECTION OF
FOETAL THORACIC DUCT LYMPHOCYTES
FOETAL THORACIC
DUCT CANNULA

B. RESULTS

5.1 INJECTION OF NON-SENSITIZED MATERNAL CELLS INTO FOETUS

The unstimulated cells used for foetal injection were popliteal efferent lymphatic lymphocytes collected from the ewe bearing that foetus (Figure 5.1). The results presented in Chapters 1 and 2 indicate that these cells had not been spontaneously sensitized by pregnancy. A variable number (7.5×10^7 to 3.2×10^9) of maternal cells were injected intravenously into 9 foetuses and the consequences are summarized in Table 5.1.

57F, 80F (estimated body weight 1.35kg from Stephenson 1959, Stephenson and Lambourne 1960). The first two foetuses (57F, 80F) received maternal cells after which there was no further surgical intervention. 57F received 7.5×10^7 maternal cells at 110 days gestation and was born 38 days later. 80F received 10^9 cells at 110 days gestation and was born 39 days later. These two lambs grew normally and no signs of wasting were observed.

The next three foetuses (41F, 23F, 50F) underwent thoracic duct cannulation and were subsequently challenged with normal maternal cells to observe the effects of this procedure on the foetus, its lymphocyte output, and the reactivity of recovered lymphocytes.

41F (estimated body weight 1.08 - 1.3kg). This animal was given a total of 3×10^9 cells over 3 days. As shown in Figure 5.2(a), thoracic duct flow ceased 12hr after the last injection and the foetus was found to be dead at laparotomy. The reactivity of foetal thoracic duct lymphocytes was not tested.

Table 5.1 Consequences of injection of unstimulated maternal cells into the foetus.

Foetus	Gestational age when challenged (days)	Cell Dose ($\times 10^9$)	Remarks
57F	110	0.075	Thoracic duct not cannulated, survived.
80F	110	1.0	Thoracic duct not cannulated, survived.
41F	109	0.5	Died 12h after the 3rd challenge.
	110	1.0	
	111	1.5	
23F	114	1.0	
	115	0.8	Survived.
	124	1.4	
50F	117	1.0	Stillbirth 13 days after challenge. No signs of graft-versus-host disease.
82F	111	1.0	Survived until the 11th day after challenge when foetal thoracic duct cannulation was unsuccessfully undertaken.
87F	112	1.0	Survived until the 17th day after challenge when foetal thoracic duct cannulation was unsuccessfully undertaken.
83F	114	1.0	Survived until the 6th day after the 2nd challenge when foetal thoracic duct cannulation was unsuccessfully undertaken.
84F	116	1.0	
	133	0.5	Survived.

Figure 5.2 Cell output in thoracic duct lymph of foetuses challenged with unstimulated maternal cells.

(a) 41F.

(b) 23F.

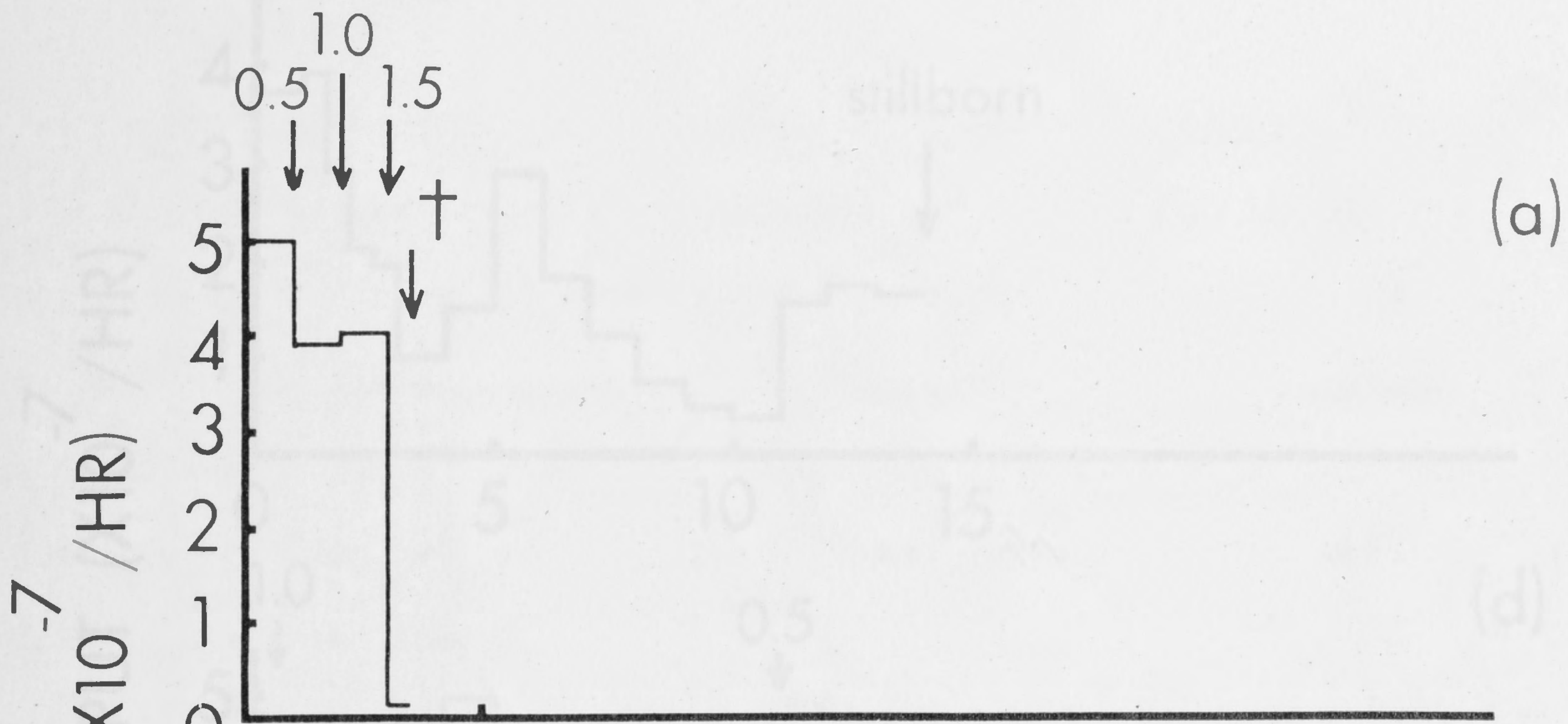
(c) 50F.

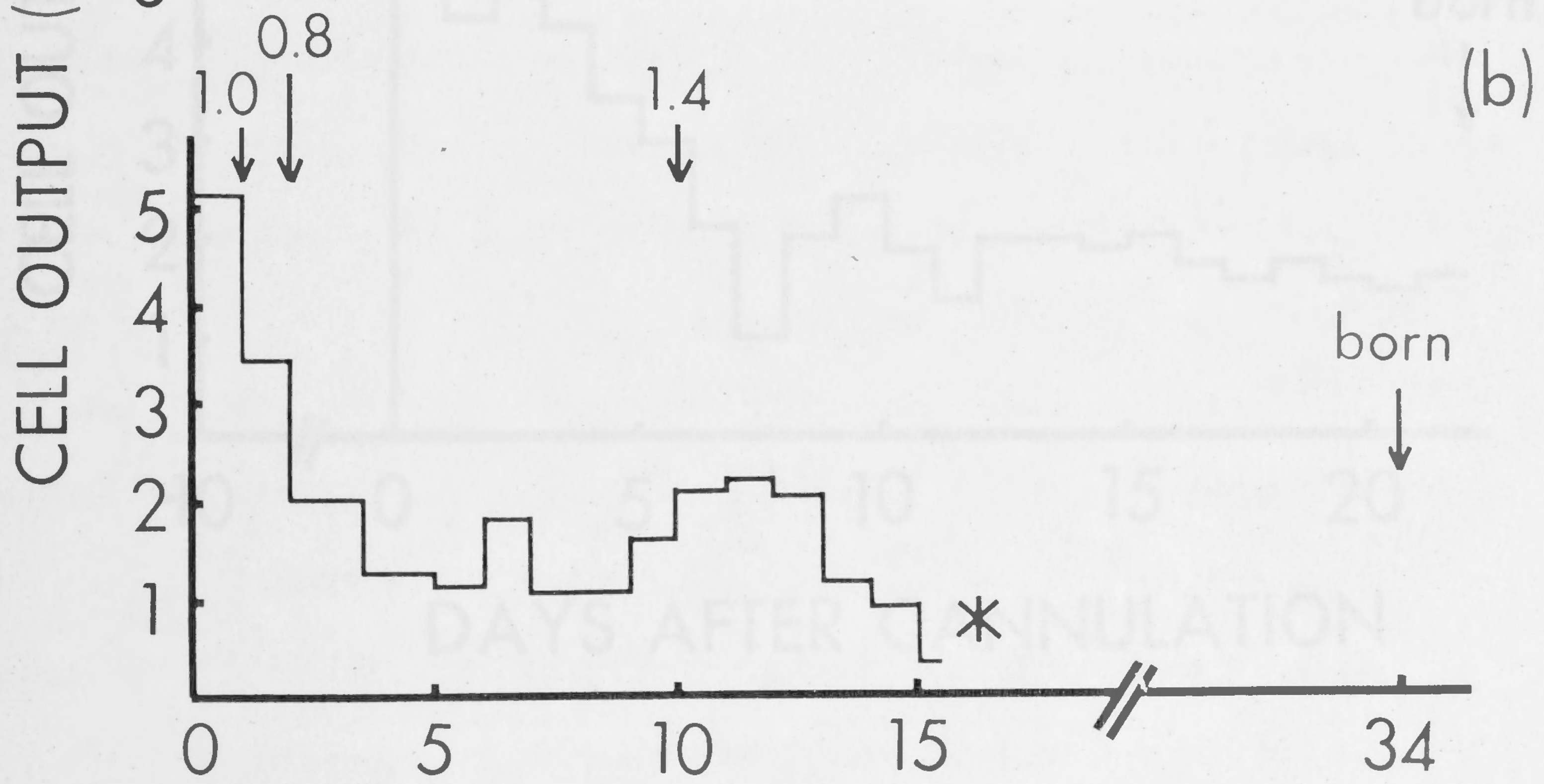
(d) 84F.

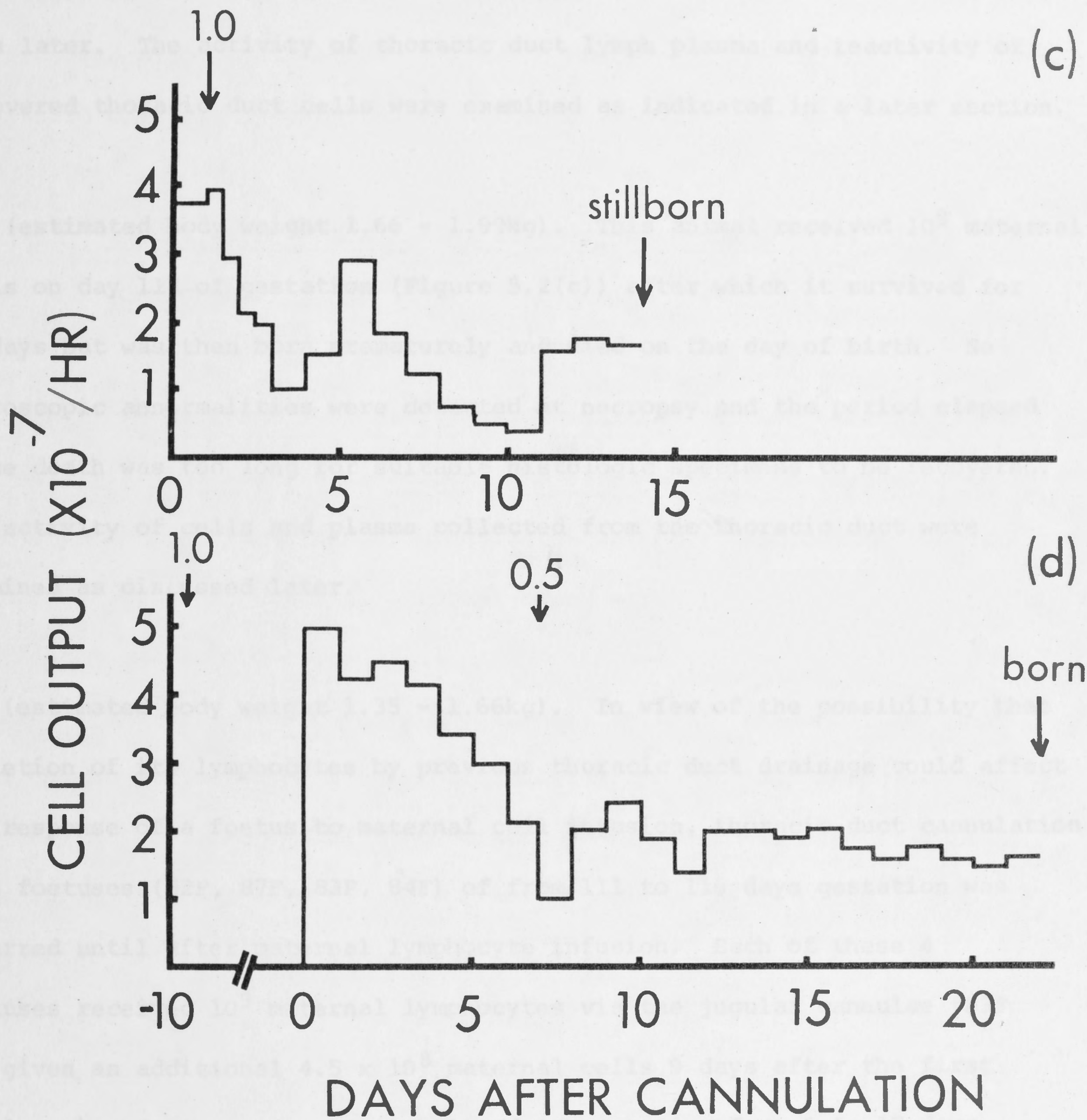
Arrows and figures on each arrow represent timing and number of maternal cells infused ($x\ 10^{-9}$) respectively.

† represents death of foetus.

* represents cessation of lymph flow from foetal thoracic duct cannula.


0.5
1.0
1.5
†
(a)
5
4
3
2
1
0


1.0
0.8
1.4
(b)
born
*
5
4
3
2
1
0
5
10
15
34
CELL OUTPUT (X10^{-7} /HR)
DAYS AFTER CANNULATION


(c)
1.0
stillborn
5
4
3
2
1
0
5
10
15
CELL OUTPUT (X10 -7 /HR)
(d)
1.0
0.5
born
-10
0
5
10
15
20
DAYS AFTER CANNULATION

23F (estimated body weight 1.65kg). Three doses of maternal cells were administered to this animal (10^9 on day 114, 0.8×10^9 on day 115 and 1.4×10^9 on day 124). The flow of thoracic duct lymph ceased on day 128 (Figure 5.2(b)) but the foetus survived and appeared normal when born 20 days later. The activity of thoracic duct lymph plasma and reactivity of recovered thoracic duct cells were examined as indicated in a later section.

50F (estimated body weight 1.66 - 1.99kg). This animal received 10^9 maternal cells on day 117 of gestation (Figure 5.2(c)) after which it survived for 13 days but was then born prematurely and died on the day of birth. No macroscopic abnormalities were detected at necropsy and the period elapsed since death was too long for suitable histologic specimens to be recovered. The activity of cells and plasma collected from the thoracic duct were examined as discussed later.

84F (estimated body weight 1.35 - 1.66kg). In view of the possibility that depletion of its lymphocytes by previous thoracic duct drainage could affect the response of a foetus to maternal cell infusion, thoracic duct cannulation of 4 foetuses (82F, 87F, 83F, 84F) of from 111 to 116 days gestation was deferred until after maternal lymphocyte infusion. Each of these 4 foetuses received 10^9 maternal lymphocytes via the jugular cannulae (83F was given an additional 4.5×10^8 maternal cells 9 days after the first challenge). Foetuses were re-submitted to operation from 6 to 17 days after lymphocyte infusion. Unfortunately, thoracic duct lymph was successfully collected from only one of these foetuses (84F). A second infusion of 5×10^8 maternal lymphocytes, given after the thoracic duct cannulation, did not produce any overt ill-effects on this foetus which was later born normally (Figure 5.2(d)). Both this lamb (84F) and the other 3 which had received maternal lymphocytes (57F, 80F, 23F) grew normally during the first 4 months of postnatal life.

These results imply that normal maternal lymphocytes may be transferred to the foetus in numbers likely to be vastly in excess of those ever likely to cross the placenta spontaneously, without noxious effect. To obtain additional information on the possible hazards of transplacental lymphocyte traffic, the effects of transfer of large numbers of specifically sensitized maternal lymphocytes into the foetus were examined.

5.2 INJECTION OF SPECIFICALLY SENSITIZED MATERNAL CELLS INTO THE FOETUS

Popliteal efferent lymphatics were cannulated in five ewes (38F, 29F, 35F, 40F, 31F) in late stages (from 106 to 130 days) of pregnancy. One to three days later, thoracic duct cannulation was performed on the foetuses of these ewes. The maternal popliteal nodes were then challenged, either with foetal thoracic duct cells or cells obtained from the ram's popliteal efferent lymphatic. There was a significant cellular response from popliteal nodes within several days of challenge. Sensitized maternal cells from this source were washed three times, re-suspended in from 5 to 10ml of Hanks' medium and injected very slowly (5 to 10min) into the foetus via its jugular cannula.

Maternal lymphocytes sensitized against foetal cells were injected into 4 foetuses (29F, 35F, 40F, 31F) while a fifth foetus (38F) received maternal cells sensitized against paternal cells (Table 5.2)

<u>38F</u> (estimated body weight 1.35kg). This animal which had recieved 8.0 x 10^9 cells over the course of 3 days, aborted 5 days after the last challenge. The macerated state of the foetus suggested that death had occurred an appreciable time before abortion. As this animal did not have an indwelling thoracic duct cannula, it was not known exactly when the foetus died.

Table 5.2 Consequences of injection of sensitized maternal cells into foetus.

Foetus	Gestational age when challenged (days)	Cell Dose ($x 10^9$)	Cells sensitized against	Remarks
38F	110	2.5	P[1)]	Aborted 5 days after 3rd challenge (macerated).
	111	3.0		
	114	2.5		
29F	116	2.5	F[2)]	Died 24h after 2nd challenge, aborted 2 days later (macerated)
	117	3.0		
35F	127	1.0	F	Aborted 1 day after challenge; mother also died (? infection).
40F	128	1.0	F	
	129	1.0		Died 12h after 4th challenge.
	130	3.5		
	131	2.5		
31F	134	6.0	F	Died within 24h of challenge.
99F	Newborn			
	+4 [3)]	2.1	P	Survived, no detrimental effect was observed.
	+5	2.1		
	+6	1.0		
	+7	0.8		

1) paternal efferent lymphatic cells.

2) foetal thoracic duct lymphocytes.

3) 4 days postpartum

Figure 5.3 Cell output in thoracic duct lymph of foetuses challenged with sensitized maternal cells.

(a) 29F.

(b) 35F.

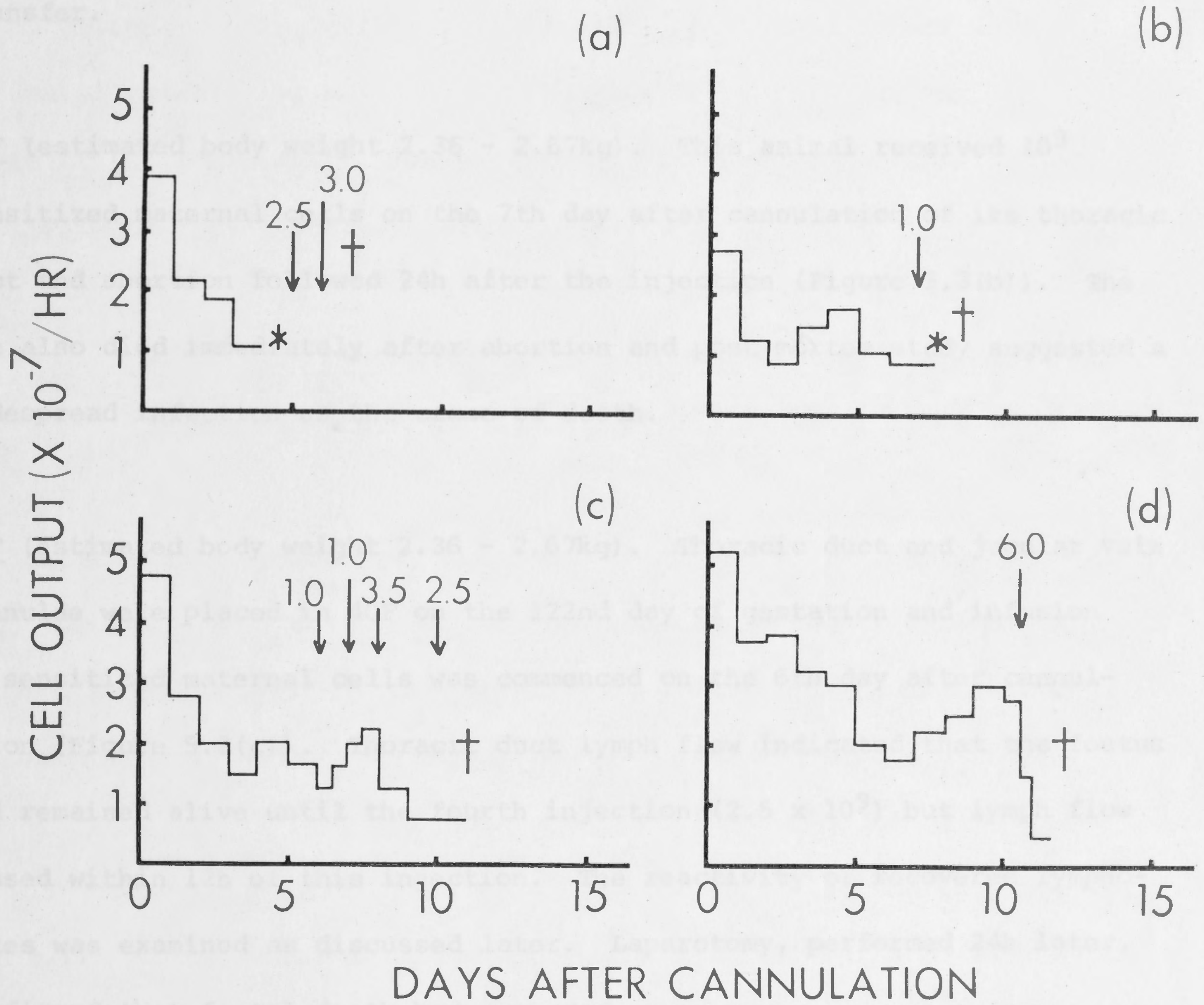
(c) 40F.

(d) 31F.

Arrows and figures on each arrow represent timing and number of maternal cells injused (x lo^{-9}).

† represents death of foetus.

* represents cessation of lymph flow from foetal thoracic duct cannula.



(a)
(b)
(c)
(d)
CELL OUTPUT (X 10^-7/HR)
DAYS AFTER CANNULATION
2.5
3.0
1.0
1.0
1.0
3.5
2.5
6.0
0
5
10
15

<u>29F</u> (estimated body weight 1.66 - 1.99kg). A total of 5.5 x 10^9 cells were infused into 29F with the result that the foetus aborted 3 days after the last transfusion. Flow from the thoracic duct cannula of this animal ceased a day before the first injection (Figure 5.3(a)), but the blood samples obtained from the jugular cannula of the foetus were used to monitor foetal viability. Foetal death was indicated by the appearance of grossly haemolysed blood in its jugular cannula a day after the last cell transfer.

<u>35F</u> (estimated body weight 2.36 - 2.67kg). This animal received 10^9 sensitized maternal cells on the 7th day after cannulation of its thoracic duct and abortion followed 24h after the injection (Figure 5.3(b)). The ewe also died immediately after abortion and post mortem study suggested a widespread infection as the cause of death.

<u>40F</u> (estimated body weight 2.36 - 2.67kg). Thoracic duct and jugular vein cannulae were placed in 40F on the 122nd day of gestation and infusion of sensitized maternal cells was commenced on the 6th day after cannulation (Figure 5.3(c)). Thoracic duct lymph flow indicated that the foetus had remained alive until the fourth injection (2.5 x 10^9) but lymph flow ceased within 12h of this injection. The reactivity of recovered lymphocytes was examined as discussed later. Laparotomy, performed 24h later, confirmed that foetal death had occurred.

<u>31F</u> (estimated body weight 3.2kg). This foetus received 6.0 x 10^9 sensitized cells on the 10th day after thoracic duct cannulation. Foetal lymph flow ceased 12h after challenge and its death was confirmed by laparotomy 24h later (Figure 5.3(d)). Usable histological samples could not be obtained because the foetal organs disintegrated rapidly after death <u>in utero</u> and no specific macroscopic features were evident at autopsy.

99F (estimated body weight 5.2kg). One ewe, the popliteal node of which had been challenged with paternal cells, produced a lamb. This lamb weighed 5.2kg whereas estimated weight of a 135 day-old foetus (31F) was 3.2kg. Challenge of this newborn over the course of the first 4 days after birth, with 6.0×10^9 sensitized cells did not compromise normal development in any discernible way.

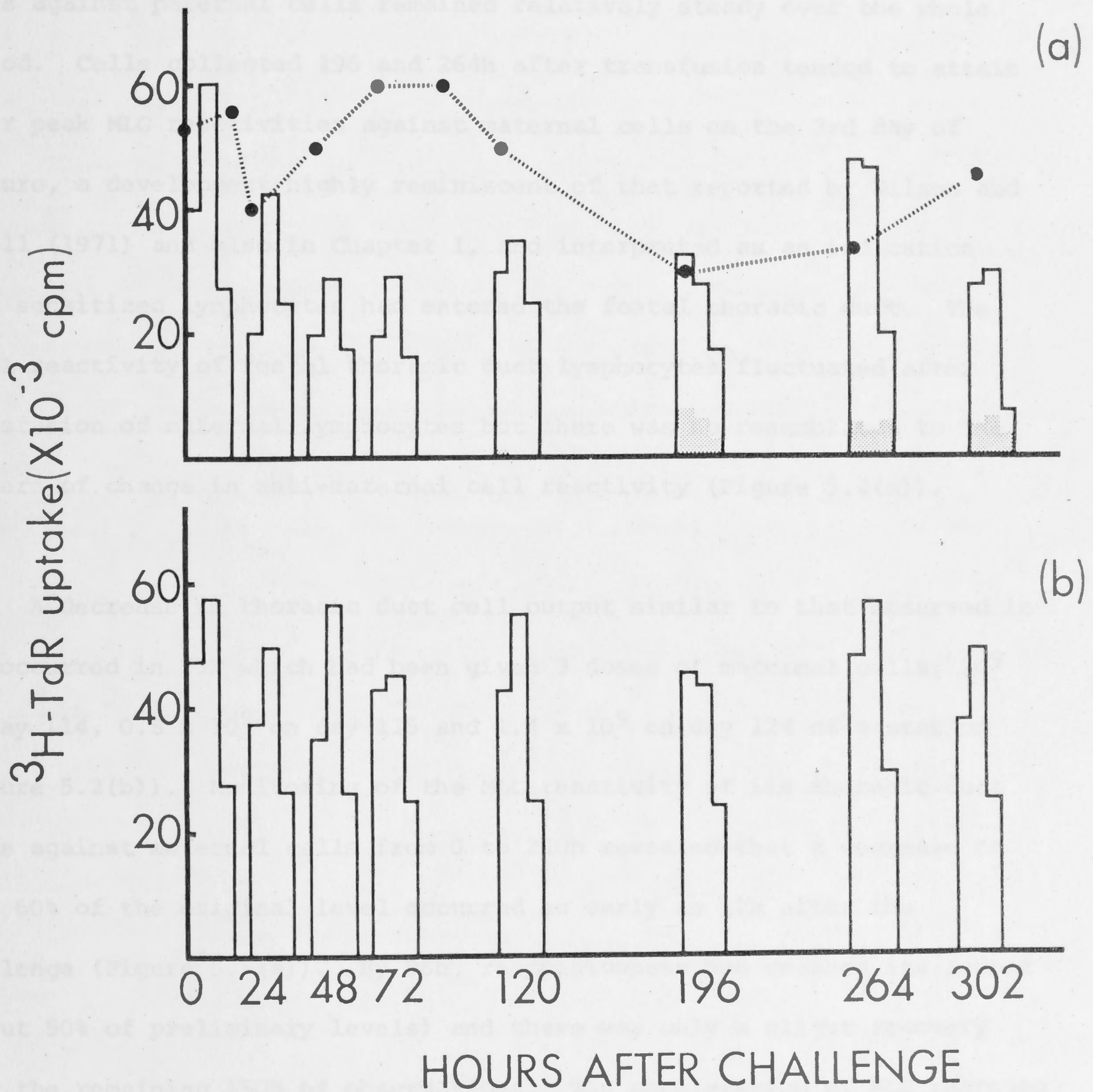
Clearly, the consequences for the foetus of infusion of large numbers of normal and sensitized maternal cells were very different, with the second procedure being universally lethal. Apart from observing the effects of transfer of maternal lymphocytes on foetal survival, the nature of the recipients' immune response was assessed by examination of reactivity of foetal lymphocytes.

5.3 REACTIVITY OF THORACIC DUCT LYMPHOCYTES COLLECTED FROM TRANSFUSED FOETUSES

Thoracic duct lymphocytes collected every 12 to 24h from 4 foetuses (50F, 23F, 40F, 84F) which had received inocula of from 1.0 to 8.0×10^9 maternal cells between 114 and 133 days of gestation were cryopreserved and their reactivity was tested *in vitro* at a common time.

50F. Thoracic duct lymphocytes were successfully collected from 50F for 302h after it had received 10^9 unstimulated maternal cells on day 117 of gestation. The output of thoracic duct cells decreased rapidly over the first 4 days of drainage (Figure 5.2(c)) as observed by Pearson, Simpson-Morgan and Morris (1976). This rapid decrease in cell output over the first 4 to 6 days of foetal thoracic duct drainage was invariably observed and was apparently uninfluenced by transfusion of maternal cells into the foetus. MLC reactivity of the collected foetal cells was measured against maternal and paternal cells (Figure 5.4(a) & 5.4(b)).

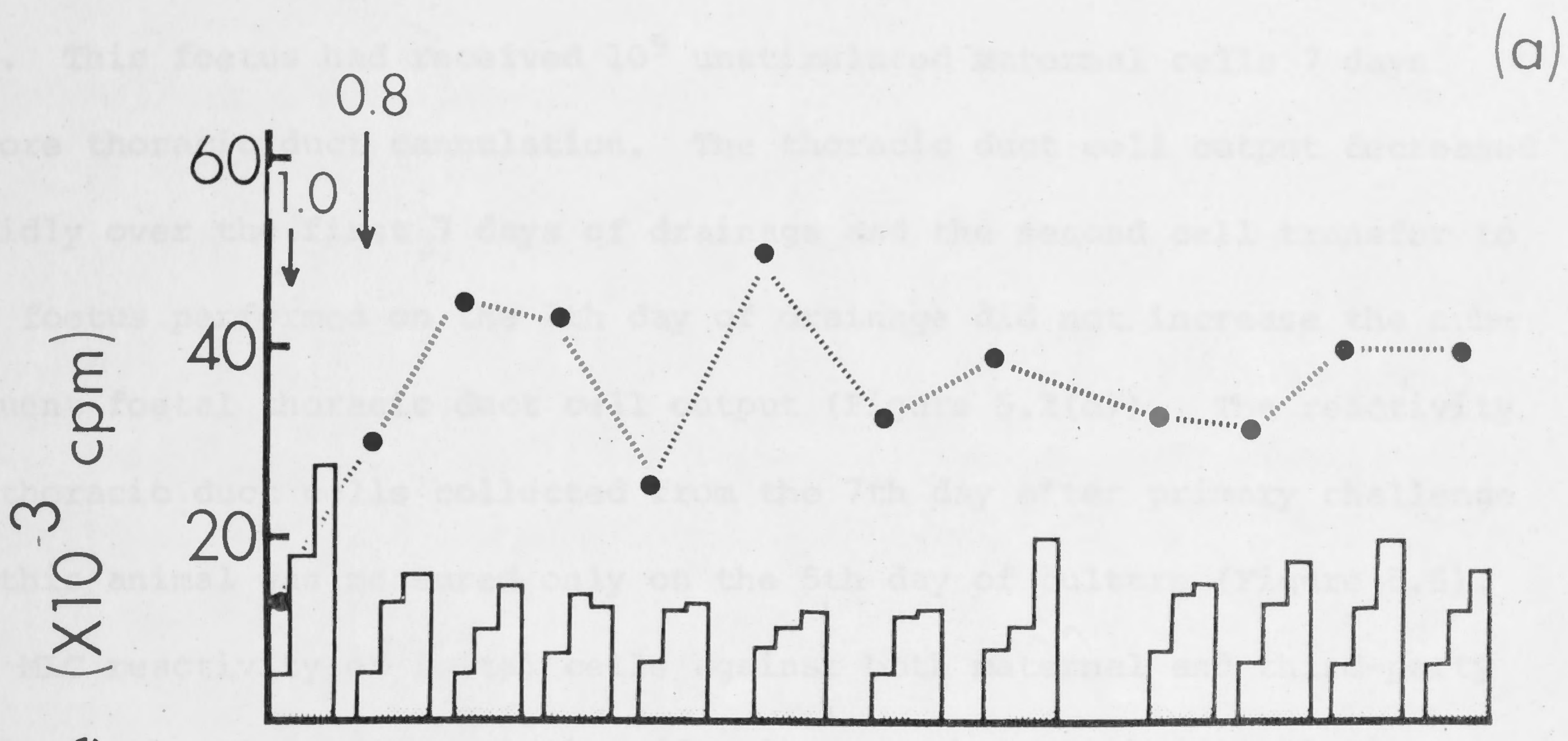
Figure 5.4 MLC and Con A reactivity of 50F foetal thoracic duct lymphocytes. 50F received i.v. injection of 10^9 unstimulated cells on 117 days gestation. Each group of 3 columns in graphs (a) and (b) represents the MLC response on days 3, 5 and 7. Graph (a) shows Con A reactivity (●) and MLC reactivity against maternal cells whereas graph (b) shows MLC reactivity against third-party cells. Results are expressed as mean cpm of triplicate cultures. Standard errors, which were always within 15% of means, were omitted for clarity. Hatched areas represent mean cpm of an autologous control. Sample collected at 0 h indicates that the sample was collected before challenge.

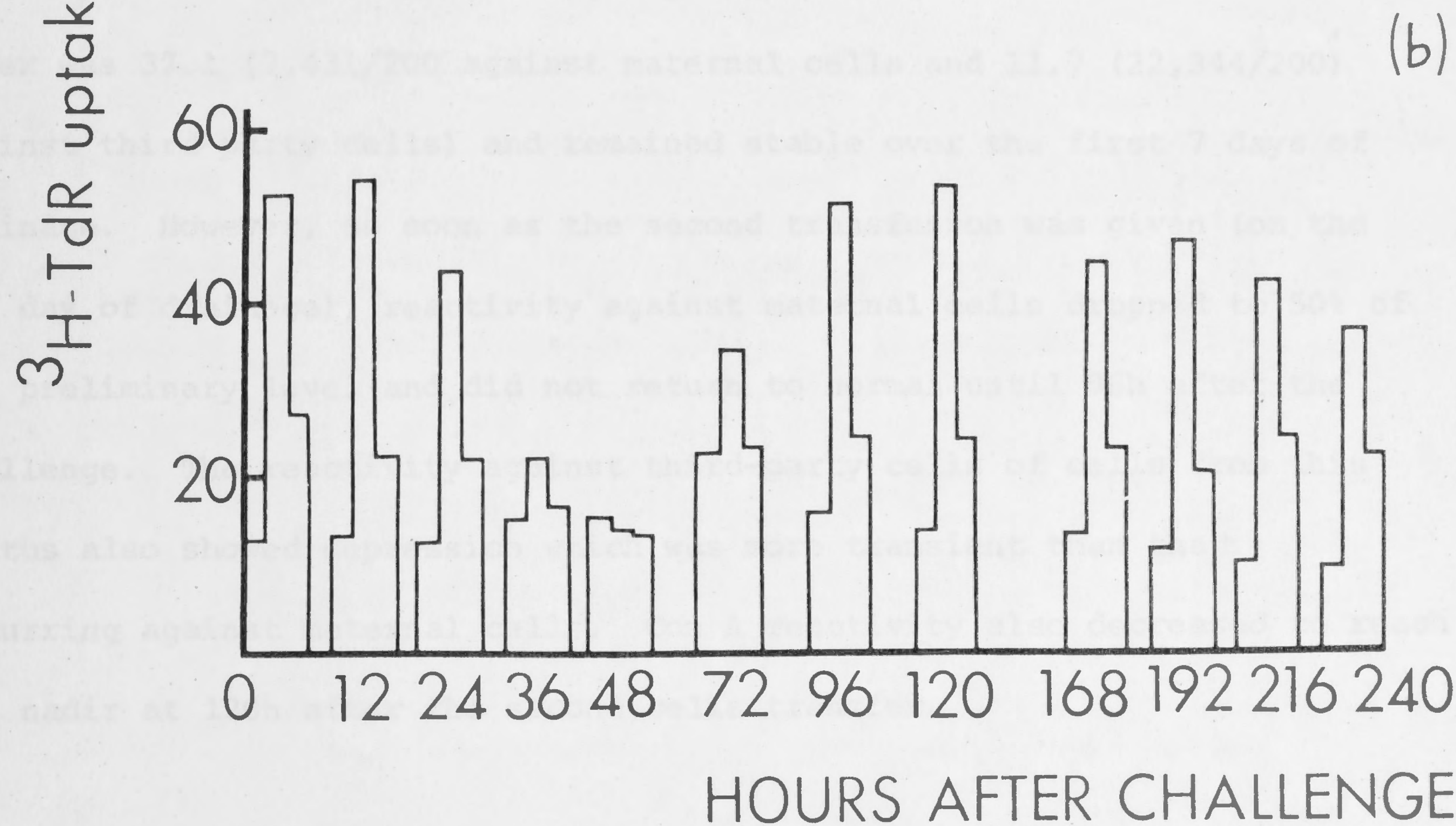

(a)
(b)
60
40
20
3H-TdR uptake(X10-3 cpm)
0
24
48
72
120
196
264
302
HOURS AFTER CHALLENGE

The response to both types of parental cells of foetal cells collected before infusion of maternal lymphocytes was normal in magnitude and tempo. Immediately after the introduction of 10^9 maternal cells into the foetus, the MLC reactivity of its thoracic duct cells against maternal cells decreased rapidly to reach a nadir between 48 and 72h after the challenge. Reactivity against maternal cells persisted at a low level for more than 100h. In contrast, MLC reactivity of foetal cells against paternal cells remained relatively steady over the whole period. Cells collected 196 and 264h after transfusion tended to attain their peak MLC reactivities against maternal cells on the 3rd day of culture, a development highly reminiscent of that reported by Wilson and Nowell (1971) and also in Chapter 1, and interpreted as an indication that sensitized lymphocytes had entered the foetal thoracic duct. The Con A reactivity of foetal thoracic duct lymphocytes fluctuated after transfusion of maternal lymphocytes but there was no resemblance to the pattern of change in anti-maternal cell reactivity (Figure 5.4(a)).

23F. A decrease in thoracic duct cell output similar to that observed in 50F occurred in 23F which had been given 3 doses of maternal cells, 10^9 on day 114, 0.8×10^9 on day 115 and 1.4×10^9 on day 124 of gestation (Figure 5.2(b)). Monitoring of the MLC reactivity of its thoracic duct cells against maternal cells from 0 to 240h revealed that a decrease to some 60% of the original level occurred as early as 12h after the challenge (Figure 5.5(a)). By 96h, responsiveness had reached its lowest (about 50% of preliminary levels) and there was only a slight recovery over the remaining 150h of observations. The acceleration of MLC response which had resulted in peak reactivity of lymphocytes collected from 50F after 196h being attained by the 3rd day of culture did not occur with 23F. MLC set up against cells from an unrelated animal revealed a drastic but comparatively ephemeral depression of reactivity between 36 and 72h after

Figure 5.5 MLC and Con A reactivity of 23F foetal thoracic duct lymphocytes. 23F received i.v. injections of 10^9 and 8×10^8 unstimulated maternal cells on day 114 and 115 respectively. Each group of 3 columns in graphs (a) and (b) represents the MLC response on days 3, 5 and 7. Graph (a) shows Con A reactivity (●) and MLC reactivity against maternal cells whereas graph (b) shows MLC reactivity against third-party cells. Results are expressed as mean cpm of triplicate cultures. Standard errors were always within 15% of means and have been omitted for clarity. The hatched area represents mean cpm of an autologous control. A sample collected at 0 h indicates that sample was collected before primary challenge. Arrows and figures on each arrow represent timing and number of maternal cells infused ($\times 10^{-9}$).


(a)
0.8
1.0
60
40
20
3H TdR uptake (X10 3 cpm)


(b)
60
40
20
0
12
24
36
48
72
96
120
168
192
216
240
HOURS AFTER CHALLENGE

the challenge with reversion of the response to normal by 96h (Figure 5.5(b)). Con A reactivity again varied irregularly without any resemblance to the course of reactivity against maternal or third-party cells (Figure 5.5(a)). The extent of any sharing of histocompatibility determinants between the third-party and maternal donors could not be determined.

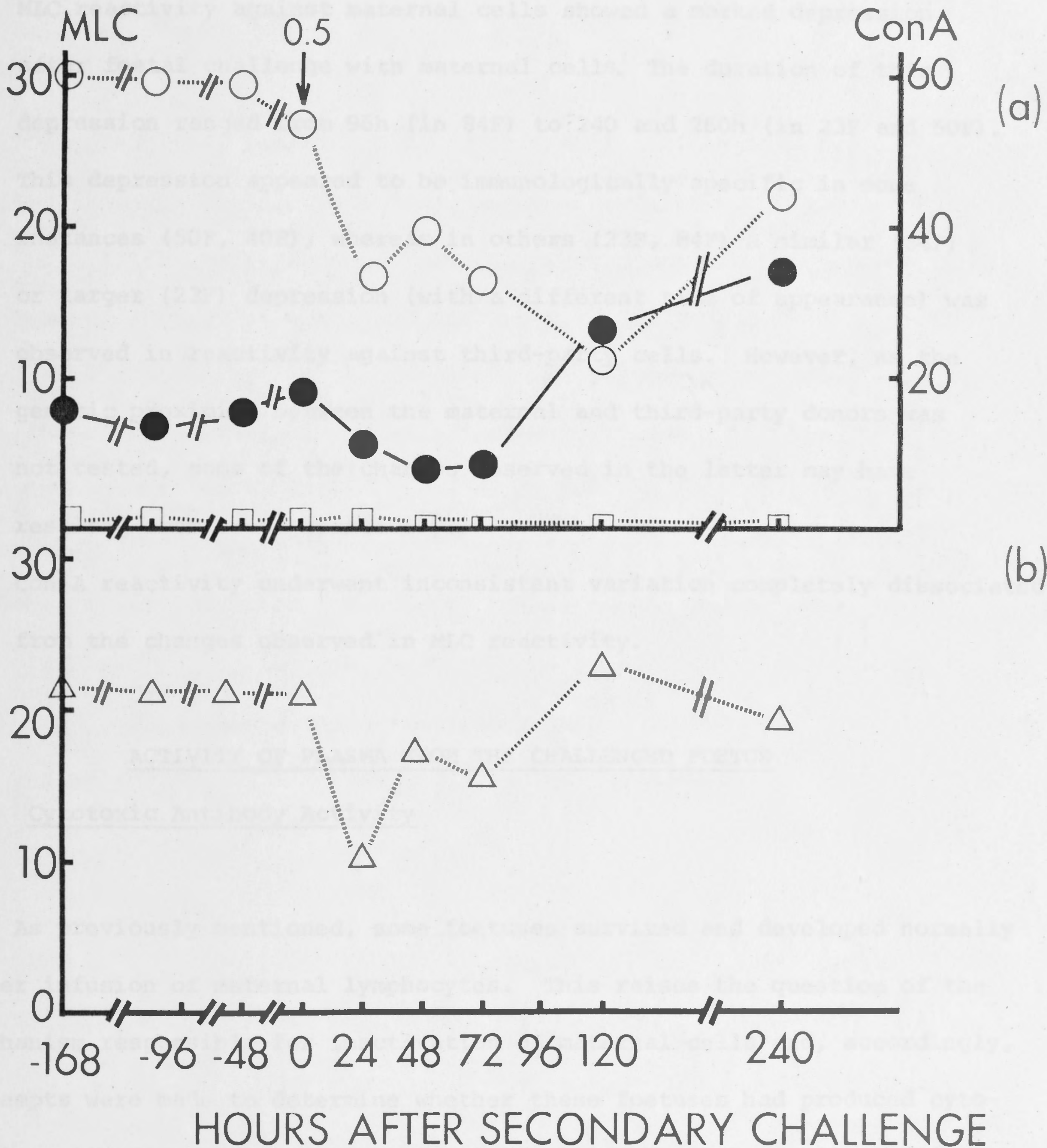
84F. This foetus had received 10^9 unstimulated maternal cells 7 days before thoracic duct cannulation. The thoracic duct cell output decreased rapidly over the first 7 days of drainage and the second cell transfer to the foetus performed on the 7th day of drainage did not increase the subsequent foetal thoracic duct cell output (Figure 5.2(d)). The reactivity of thoracic duct cells collected from the 7th day after primary challenge of this animal was measured only on the 5th day of culture (Figure 5.6). The MLC reactivity of foetal cells against both maternal and third-party cells at the time of cannulation (day 0) was substantial (stimulation index was 37.1 (7,431/200 against maternal cells and 11.7 (22,344/200) against third-party cells) and remained stable over the first 7 days of drainage. However, as soon as the second transfusion was given (on the 7th day of drainage), reactivity against maternal cells dropped to 50% of the preliminary level and did not return to normal until 96h after the challenge. The reactivity against third-party cells of cells from this foetus also showed depression which was more transient than that occurring against maternal cells. Con A reactivity also decreased to reach its nadir at 120h after the second cells transfer.

40F. This foetus received injections of sensitized maternal cells on 4 successive days starting at the 128th day of gestation (total cell dose 8.0×10^9). Although these injections produced little effect on the cell output from the foetal thoracic duct (Figure 5.3(c)), MLC reactivity of foetal against maternal cells decreased significantly over the ensuing

Figure 5.6 MLC and Con A reactivity of 84F foetal thoracic duct lymphocytes. 84F received i.v. injections of 10^9 and 5×10^8 unstimulated maternal cells on day 116 and 133 respectively. Reactivity was tested against:

Con A (○),
irradiated autologous thoracic duct cells (□),
irradiated maternal PBL (●),
irradiated third-party PBL (△).

Left ordinate indicates scale of responsiveness to MLC and right ordinate that to Con A. Results are expressed as mean cpm of triplicate cultures harvested on day 5. 0 indicates the time when a secondary challenge (5×10^8 cells) was given.


MLC
0.5
ConA
30
20
10
60
40
20
(a)
30
20
10
0
(b)
-168
-96
-48
0
24
48
72
96
120
240
HOURS AFTER SECONDARY CHALLENGE

72h (Figure 5.7(a)). However, reactivity against third-party cells remained remarkably stable (Figure 5.7(b)). Con A reactivity dropped to 30% of its original level in the 12h after challenge but then gradually recovered (Figure 5.7(a)).

In summary, the main features of the responses in foetal lymphocyte reactivity to transfusion of maternal cells were:

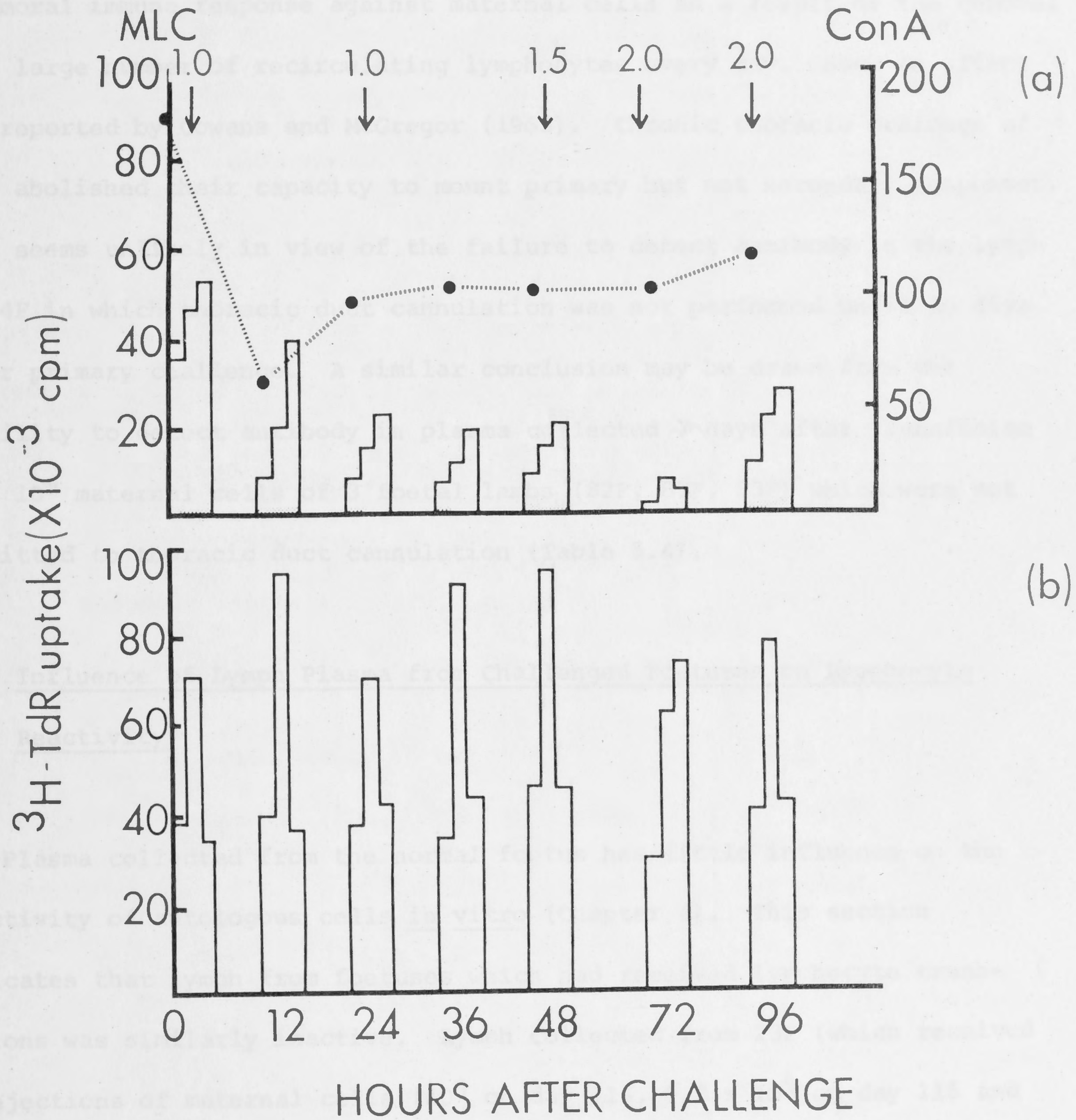
1. MLC reactivity against maternal cells showed a marked depression after foetal challenge with maternal cells. The duration of this depression ranged from 96h (in 84F) to 240 and 260h (in 23F and 50F).
2. This depression appeared to be immunologically specific in some instances (50F, 40F), whereas in others (23F, 84F) a similar (84F) or larger (23F) depression (with a different time of appearance) was observed in reactivity against third-party cells. However, as the genetic proximity between the maternal and third-party donors was not tested, some of the changes observed in the latter may have resulted from cross-reactivity.
3. Con A reactivity underwent inconsistent variation completely dissociated from the changes observed in MLC reactivity.

5.4 ACTIVITY OF PLASMA FROM THE CHALLENGED FOETUS

(a) Cytotoxic Antibody Activity

As previously mentioned, some foetuses survived and developed normally after infusion of maternal lymphocytes. This raises the question of the mechanism responsible for inactivation of maternal cells and, accordingly, attempts were made to determine whether these foetuses had produced cytotoxic antibodies. Twenty-six thoracic duct lymph samples collected from 4 foetuses following maternal cell infusion were tested for cytotoxic activity using maternal PBL as target cells in the presence of either fresh guinea pig or rabbit serum as a source of complement. As shown in

Figure 5.7 MLC and Con A reactivity of 40F foetal thoracic duct lymphocytes. 40F received 4 injections of sensitized maternal cells (10^9 on day 128, 10^9 on day 129, 3.5 x 10^9 on day 130 and 2.5 x 10^9 on day 131). Each group of 3 columns in graphs (a) and (b) represents the MLC response on days 3, 5 and 7. Graph (a) shows Con A reactivity (●) and MLC reactivity against maternal cells whereas graph (b) shows MLC reactivity against third-party cells. Results are expressed as mean cpm of triplicate cultures. Standard errors were always within 15% of means and have been omitted for clarity. The hatched area represents mean cpm of an autologous control. A sample collected at 0 indicates that the sample was collected before primary challenge. Arrows and figures on each arrow represent timing and number of maternal cells infused ($x10^{-9}$).


MLC
ConA
1.0
1.0
1.5
2.0
2.0
(a)
100
80
60
40
20
200
150
100
50
3H-TdR uptake (X10-3 cpm)
(b)
100
80
60
40
20
0
12
24
36
48
72
96
HOURS AFTER CHALLENGE

in Table 5.3, no cytotoxicity was observed in any of the lymph samples. Although thoracic duct drainage has been reported to produce only minimal effects on the lymphocyte content of the various foetal lymphoid tissues (Pearson, Simpson-Morgan and Morris 1976), the possibility remains that this procedure may have compromised effective initiation of a humoral immune response against maternal cells as a result of the removal of a large number of recirculating lymphocytes every day. Such an effect was reported by Gowans and McGregor (1963). Chronic thoracic drainage of rats abolished their capacity to mount primary but not secondary responses. This seems unlikely in view of the failure to detect antibody in the lymph of 84F in which thoracic duct cannulation was not performed until 10 days after primary challenge. A similar conclusion may be drawn from the inability to detect antibody in plasma collected 7 days after transfusion with 10^9 maternal cells of 3 foetal lambs (82F, 87F, 83F) which were not submitted to thoracic duct cannulation (Table 5.4).

(b) Influence of Lymph Plasma from Challenged Foetuses on Lymphocyte Reactivity

Plasma collected from the normal foetus has little influence on the reactivity of autologous cells *in vitro* (Chapter 4). This section indicates that lymph from foetuses which had received lymphocyte transfusions was similarly inactive. Lymph collected from 23F (which received 3 injections of maternal cells (10^9 on day 114, 0.8×10^9 on day 115 and 1.4×10^9 on day 124) was tested for its ability to affect specific and nonspecific alloreactivity of foetal cells (Figure 5.8). Apart from a lymph sample collected 24h after the first transfusion which seemed to possess some inhibitory effect, none of 11 other lymph samples modified the alloreactivity of autologous cells in any way. The ability of these 12 lymph samples to suppress maternal alloreactivity was also tested

Table 5.3 Cytotoxic activity of thoracic duct lymph collected from foetuses transfused with maternal lymphocytes.

	% Cytotoxicity									
	Time after the primary challenge (hours)									
	0	48	96	120	144	192	240	264	302	360
50F	0	0	0	0	0	0	0	0	0	NS 1)
23F	0	0	0	0	0	0	0	0	0	NS
40F	0	0	0			no samples				
84F		no samples			0 2)	n.d	0	0	0	0

1) no samples.

2) plasma was obtained from a jugular cannula and tested.

3) 50F received 10^9 unstimulated maternal cells on day 117.

4) 23F received 3 injections of unstimulated maternal cells (10^9 on day 114, 8×10^8 on day 115 and 1.4×10^9 on day 124).

5) 40F received 4 injections of sensitized maternal cells (10^9 on day 128 and day 129, 3.5×10^9 on day 130 and 2.5×10^9 on day 131) and died approximately 12h after the 4th challenge.

6) 84F received 10^9 on day 116 and was submitted to a thoracic duct cannulation on day 126. A secondary challenge was made on day 133.

7) target cells were maternal PBL.

8) both fresh guinea pig and rabbit sera were used as complement source in each assay.

Table 5.4 Cytotoxic activity of plasma collected from transferred foetuses.

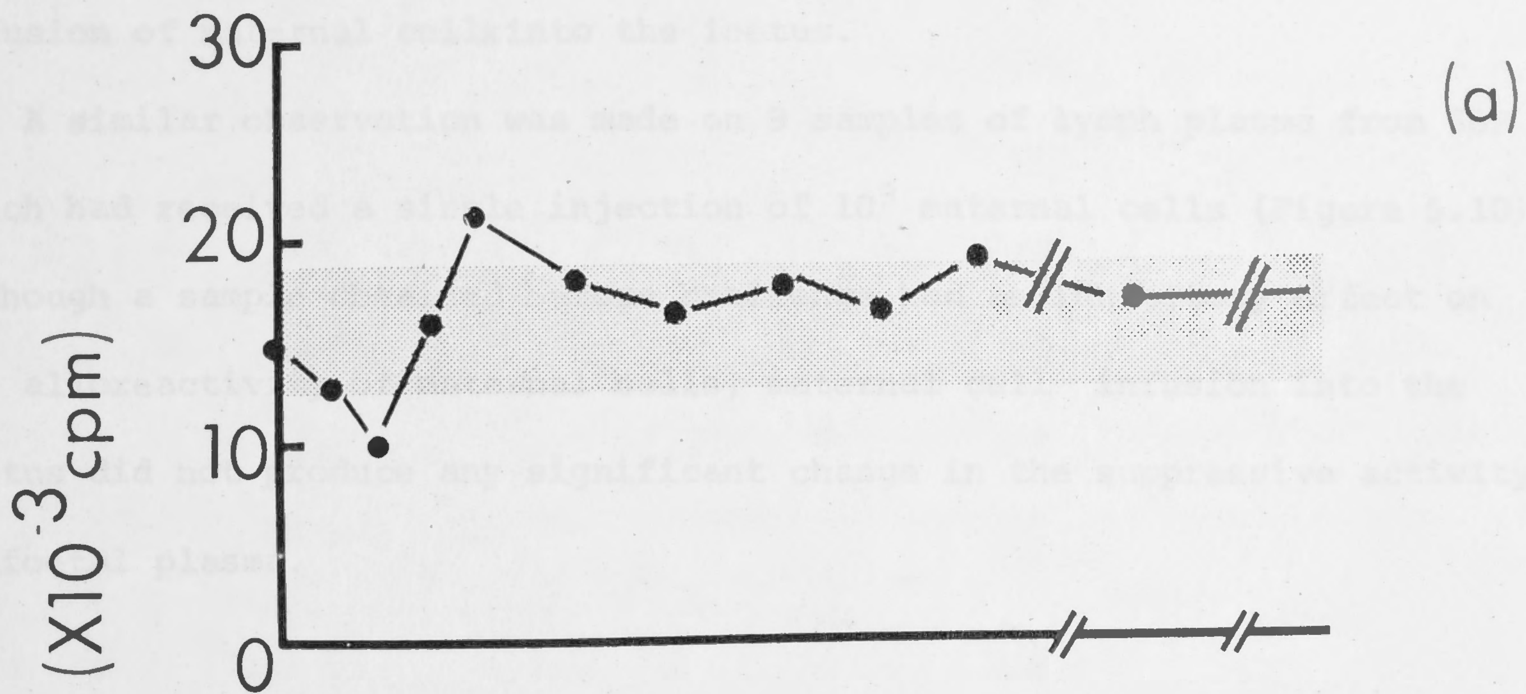
Foetus	Gestational age when challenged (days)	Time (after transfusion) of plasma collection	% Cytotoxicity against matched cells
82F	111	day 0	0
		day 7	0
87F	112	day 0	0
		day 7	0
83F	114	day 0	0
		day 7	0

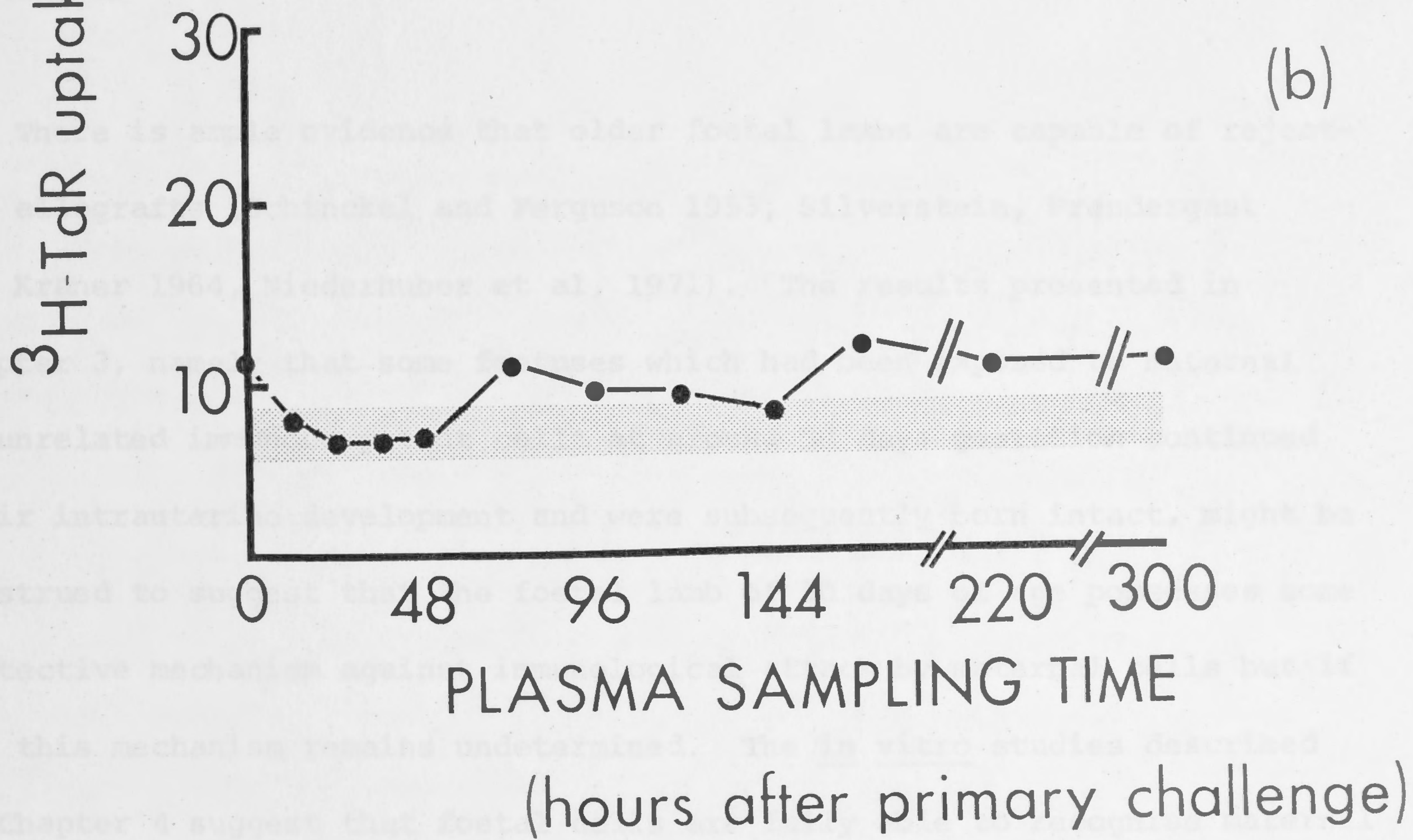
1) 82F, 87F and 83F received 10^9 unstimulated maternal cells in utero.

2) target cells were maternal PBL.

3) both fresh guinea pig and rabbit sera were used as a complement source.

Figure 5.8 Effect of foetal plasma (23F) on reactivity of autologous cells. The hatched area indicates mean cpm ± 3 S.D. of foetal response against irradiated maternal PBL in graph (a), and irradiated paternal PBL in graph (b) in the absence of foetal plasma from the culture. Responder cells were 23F foetal thoracic duct lymphocytes collected before challenge and a lymph sample collected at the designated time was introduced into the culture so as to constitute 10% of the culture medium.


30
20
10
0
(a)
3H-TdR uptake (X10-3 cpm)


30
20
10
(b)
0
48
96
144
220
300
PLASMA SAMPLING TIME
(hours after primary challenge)

(Figure 5.9). Although all of these samples decreased maternal cell reactivity against paternal cells in particular, the significance of this observation is not clear given that serum or plasma from normal (non-pregnant) ewes has been found to be suppressive to the same extent (see Figure A in Materials and Methods). However, this inhibitory activity did not appear to increase above its base level in response to the infusion of maternal cells into the foetus.

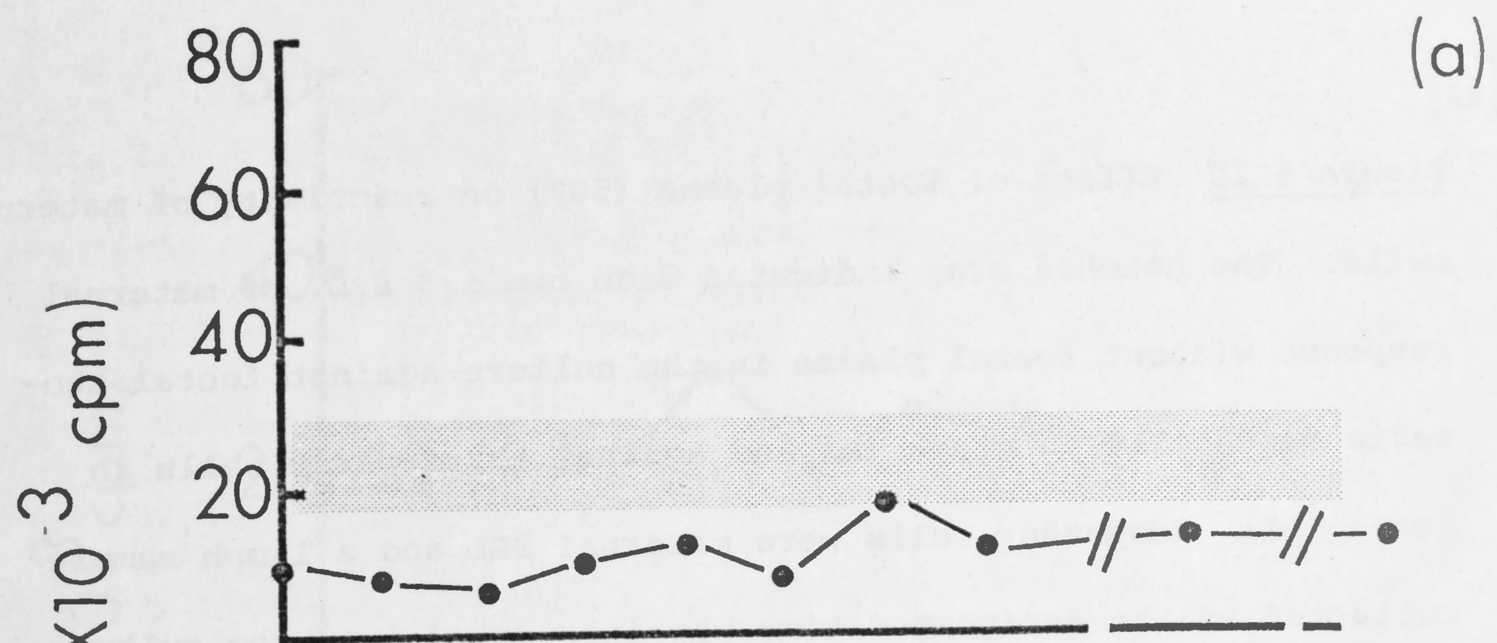
A similar observation was made on 9 samples of lymph plasma from 50F which had received a single injection of 10^9 maternal cells (Figure 5.10). Although a sample obtained before challenge had an inhibitory effect on the alloreactivity of maternal cells, maternal cell infusion into the foetus did not produce any significant change in the suppressive activity of foetal plasma.

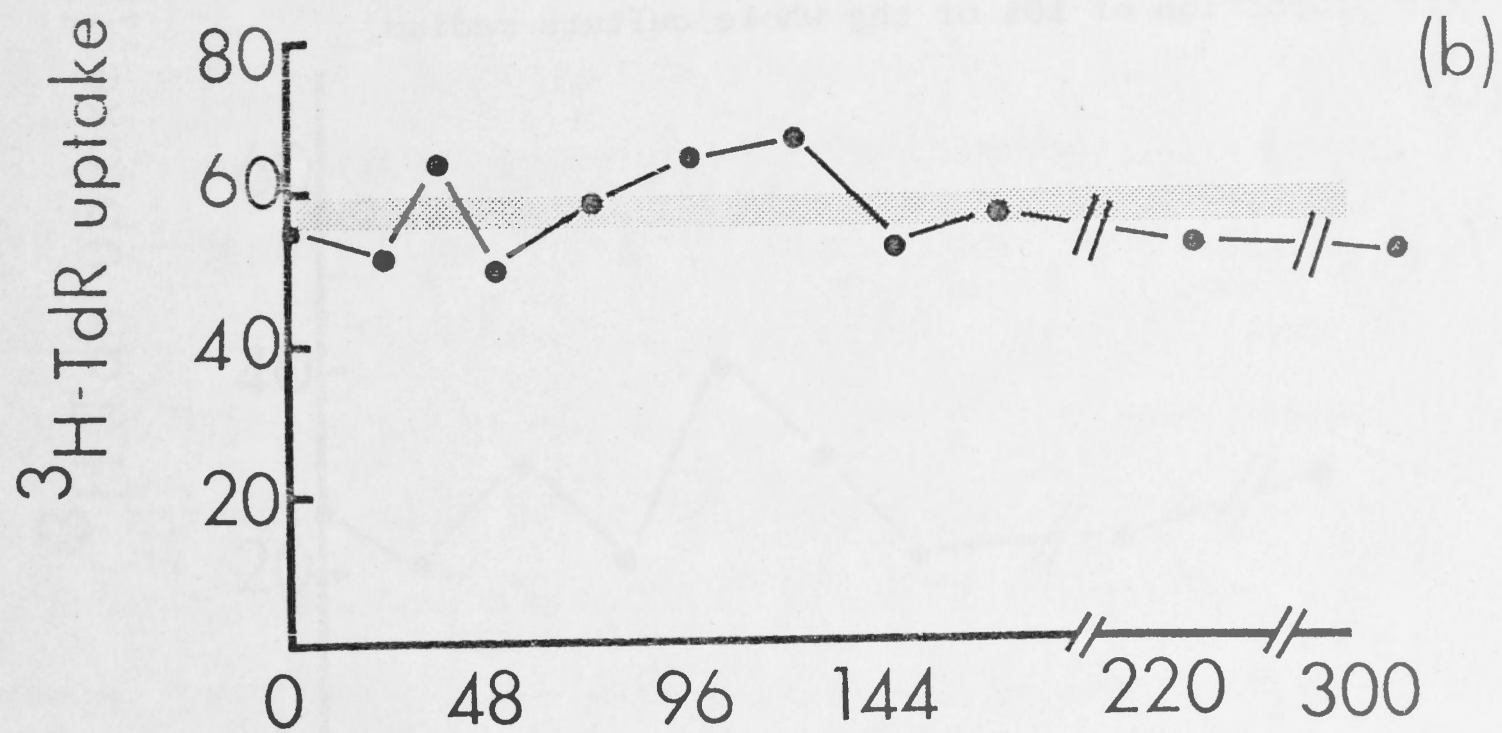
DISCUSSION

There is ample evidence that older foetal lambs are capable of rejecting allografts (Schinckel and Ferguson 1953, Silverstein, Prendergast and Kraner 1964, Niederhuber et al, 1971). The results presented in Chapter 3, namely that some foetuses which had been exposed to maternal or unrelated immunocompetent cells at around 50 days gestation continued their intrauterine development and were subsequently born intact, might be construed to suggest that the foetal lamb of 50 days of age possesses some protective mechanism against immunological attack by maternal cells but if so, this mechanism remains undetermined. The *in vitro* studies described in Chapter 4 suggest that foetal cells are fully able to recognise maternal cells as foreign and that this is likely to represent the *in utero* situation accurately.

The experiments reported in the present chapter were designed to study the capacity of the foetus to respond to challenge with very large numbers of maternal cells.

Figure 5.9 Effect of foetal plasma (23F) on reactivity of maternal cells. The hatched area indicates mean cpm ± 3 S.D. of maternal response without foetal plasma in culture against irradiated paternal cells in graph (a) and against irradiated third-party cells in graph (b). Responder cells were maternal popliteal efferent lymphatic lymphocytes and a lymph sample collected at the designated time was introduced into the culture in the proportion of 10% of the whole culture medium.





^{3}H-TdR uptake (X10^{-3} cpm)

PLASMA SAMPLING TIME

(hours after primary challenge)

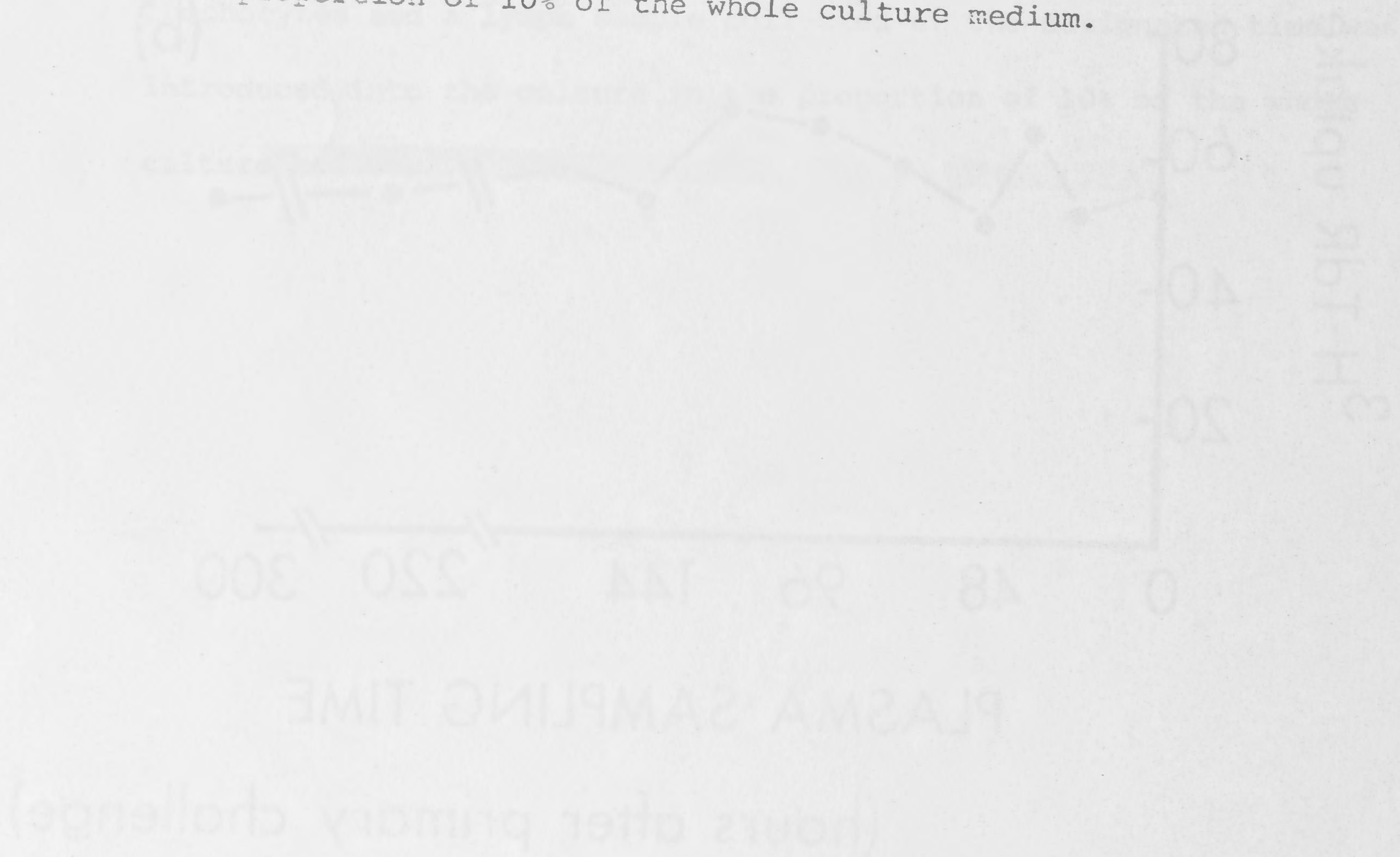
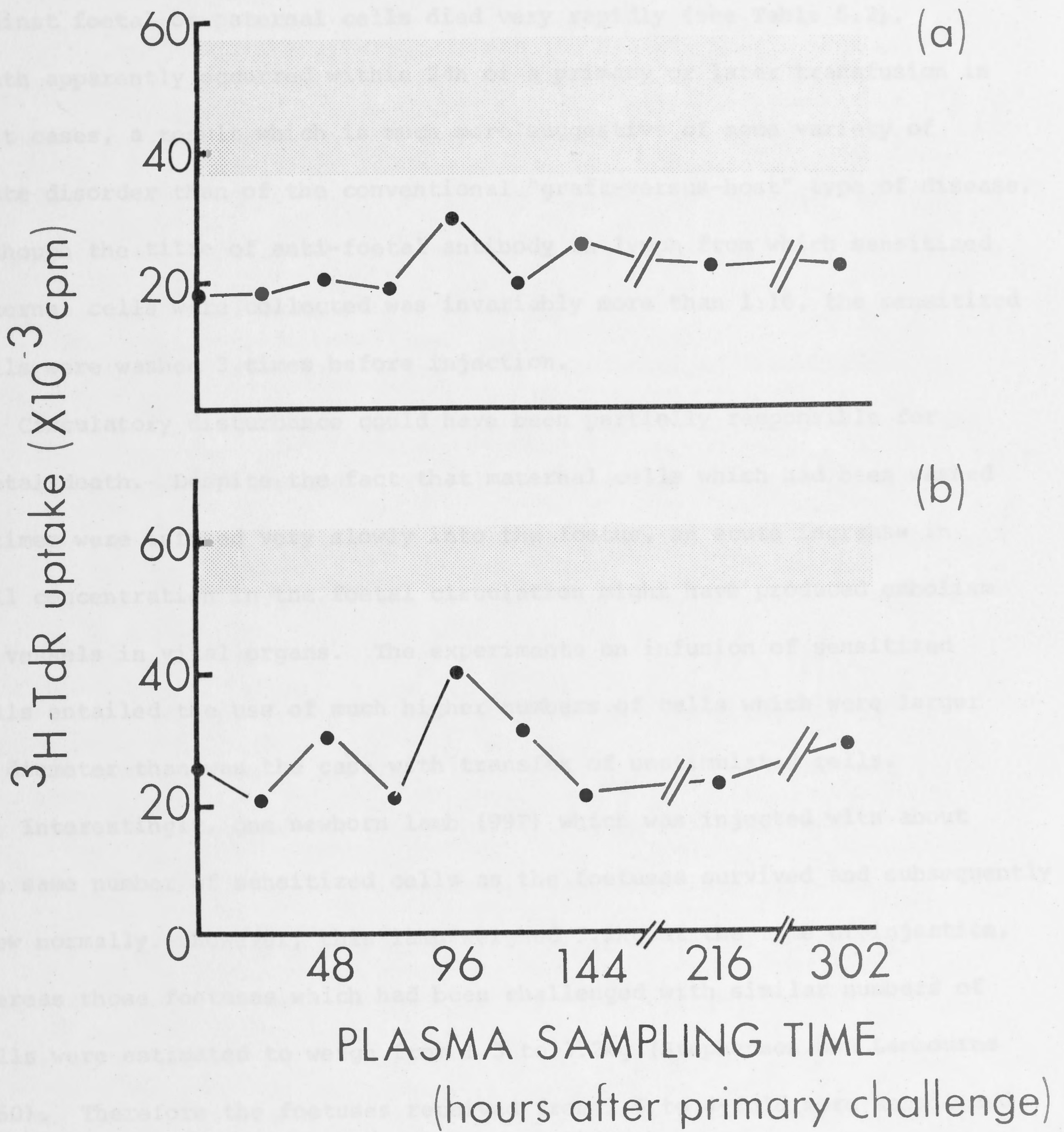


Figure 5.10 Effect of foetal plasma (50F) on reactivity of maternal cells. The hatched area indicates mean cpm ± 3 S.D. of maternal response without foetal plasma in the culture against foetal thoracic duct cells in graph (a) and against third-party cells in graph (b). Responder cells were maternal PBL and a lymph sample collected at the designated time was introduced into the culture in the proportion of 10% of the whole culture medium.


(a)
(b)
60
40
20
0
3H TdR uptake (X10-3 cpm)
48
96
144
216
302
PLASMA SAMPLING TIME
(hours after primary challenge)

Four foetuses (57F, 80F, 23F, 84F) which had received large numbers of unstimulated maternal cells in utero survived to term (see Table 5.1) and showed no pathological signs suggesting that the foetus is able to kill or inactivate maternal cells in utero. However, all foetuses that received large numbers of maternal lymphocytes specifically sensitized against foetal or paternal cells died very rapidly (see Table 5.2). Death apparently occurred within 24h of a primary or later transfusion in most cases, a result which is much more suggestive of some variety of acute disorder than of the conventional "graft-versus-host" type of disease. Although the titre of anti-foetal antibody in lymph from which sensitized maternal cells were collected was invariably more than 1:16, the sensitized cells were washed 3 times before injection.

Circulatory disturbance could have been partially responsible for foetal death. Despite the fact that maternal cells which had been washed 3 times were infused very slowly into the foetus, an acute increase in cell concentration in the foetal circulation might have produced embolism of vessels in vital organs. The experiments on infusion of sensitized cells entailed the use of much higher numbers of cells which were larger in diameter than was the case with transfer of unstimulated cells.

Interestingly, one newborn lamb (99F) which was injected with about the same number of sensitized cells as the foetuses survived and subsequently grew normally. However, this lamb weighed 5.2kg at the time of injection, whereas those foetuses which had been challenged with similar numbers of cells were estimated to weigh from 1.3 to 3.2kg (Stephenson and Lambourne 1960). Therefore the foetuses received from 1.5 to 4-fold more sensitized cells per kilogram body weight than the newborn lamb. Unfortunately, blockage of the maternal popliteal cannula prevented further injection of sensitized cells into this lamb.

It is probable that the numbers of cells used for foetal infusions in this study were unphysiologically high. In some experiments, as many

as 3 to 6 x 10^9 cells were infused at one time and these numbers are close to the recirculating lymphocyte pool of the foetus between 130 and 135 days of gestation (Pearson, Simpson-Morgan and Morris 1976) and far in excess of those which current knowledge of transplacental traffic would indicate to be likely to occur normally (Brambell 1970). However, the objective of the present experiment was to expose the capabilities of any mechanisms responsible for maintaining foetal integrity in the face of possible immunological aggression by the mother. Furthermore, given the impracticability of testing foetal responses to a wide range of maternal cell numbers, it was considered that the use of the most extreme challenge procedure could be the most informative.

The surgical stress associated with cannulation of foetal vessels has to be considered as a cause of foetal death but the demonstration by Pearson, Simpson-Morgan and Morris (1976) that foetal thoracic duct drainage had minimal effects on subsequent development and growth suggests that the stress of surgery would have had little effect on the course of the present experiment.

The failure to detect cytotoxic antibody in challenged foetuses requires comment. It is possible that antibodies against maternal cells may have been formed by the foetus but failed to fix complement as reported by Bell (1980) of alloantibodies from multiparous mice. Absorption of antibody activity onto transfused maternal cells could have been a factor in the case of multiple transfusions, but not after a single transfusion.

The failure of plasma collected from challenged foetuses to inhibit the _in vitro_ alloreactivity of autologous cells against maternal cells suggests that the reactivity of such foetal cells in the foetal circulation was likely to be similar to that observed _in vitro_. The necessity for caution in interpretation of the effects of plasma on lymphocyte reactivity _in vitro_ is indicated by the depression of MLC

reactivity produced by plasma from ewes before pregnancy, as discussed in Chapter 1.

The survival of 4 foetuses after *in utero* transfusion with very large numbers of normal maternal cells suggests that the foetus is able to kill or inactivate maternal cells. It is not feasible, from the present observations, to distinguish between these two possible outcomes. While destruction of the foreign maternal cells would be the more likely, the operation of a mechanism similar to that reported to be responsible for selective inactivation of those maternal lymphocytes reactive against foetal and neonatal F_1 hybrid rats remains possible (McCullagh 1977, 1980a). Distinction between the 2 possibilities would require positive identification of maternal cells with reactivities other than antifoetal in the circulation of the foetus.

The dramatic change in the reactivity of thoracic duct lymphocytes that followed foetal challenge with maternal cells requires comment. A similar change of reactivity was observed in pregnant ewes challenged with foetal or paternal cells (see Chapter 2). Maternal cells collected from the stimulated lymph node showed a transient but significant, and apparently nonspecific, depression of reactivity. On the other hand, the depression of reactivity observed in 2 foetal animals (50F, Figure 5.4; 40F, Figure 5.7) appeared to be specific. Presumably, the depression observed in ewe and foetus, after mutual challenge, represents the phenomenon described in non-pregnant sheep by Hay, Cahill and Trnka (1974) and most commonly ascribed to selective removal of specifically reactive lymphocytes from circulation.

The present demonstration that this phenomenon occurs in the foetus implies, as do many of the other experiments in this study, a considerable degree of immunological maturation. It may also indicate a response of the foetus to maternal lymphocytes that has potential usefulness for the foetus.

GENERAL DISCUSSION

The primary aim of the present study was to document any modifications in the cellular aspects of the immunological responsiveness of the pregnant ewe to paternal or foetal antigens. Although there have been numerous investigations of the impact of parity on cellular (Beer and Billingham 1972, Maroni and De Sousa 1973, Carr, Stites and Fudenberg 1973) and humoral (Van Rood, Eernisse and Leeuwen 1958, Payne and Rolfs 1958, Herzenberg and Gonzales 1962, Kaliss and Dagg 1964) types of immune responses in a variety of species, longitudinal studies of responses during a first pregnancy have been uncommon. Unless the effects of a first pregnancy are studied, it is not possible to differentiate between the effects of pregnancy *per se* and the consequences of the large scale transfer of foetal cells to the mother which frequently accompanies parturition.

A second objective was to test the capacity of mother and foetus to respond to deliberate challenge with the lymphocytes of the other. Although the response of mother and foetus to grafts of solid tissue from the other had been examined it was considered that, irrespective of the placental permeability of the species, the opportunity for direct mutual exposure of circulating haematogenous cells from mother and foetus should exceed that for access by circulating cells from either party to sessile cells in the tissues of the other. The rejection of foetal limb grafts by pregnant rats (Woodruff 1958) and of maternal skin by foetal sheep (Schinckel and Ferguson 1953) did not indicate that the capacity for mutual stimulation by lymphoid cells was normal. The extent of the possible difference between reactivity of lymphocytes against allogeneic lymphoid and non-lymphoid tissues after *in utero* exposure is illustrated by the retention of primary, and of the capacity to acquire secondary, reactivity against co-twin skin by lymphocytes from twin calves which are peripheral blood leucocyte chimaeras completely unreactive to each other in mixed lymphocyte culture (Emery and McCullagh 1980b).

The third objective was to assess the likelihood that any changes observed in the immunological reactivity of the pregnant ewe could be relevant for the

successful completion of gestation. It was appreciated at the outset that the considerable interspecies differences in such details as duration of pregnancy (and consequently the period available for occurrence of maternal immune responses), placental structure (with its implications for ease of traffic of cells and antibodies) and stage of immunological maturation attained by the foetus in utero would militate against formulation of any proposals with widespread applicability.

While selection of the sheep as the experimental animal conferred obvious advantages for the conduct of foetal surgery, its outbred status introduced some limitations. The most restrictive of these arose from inability to measure genetic proximity between animals by histocompatibility typing. This impediment prevented the identification of any instances in which sharing of a haplotype between mother and foetus could have resulted in a major reduction of reactivity in either direction. Fortunately, the number of occasions on which low reactivity was observed were so few as to ensure that this potential difficulty did not cause concern. Another obvious limitation imposed by the use of an outbred species was that study of second pregnancies became impracticable as the genetic relationship between the two foetuses would be unknown. The most noticeable complication of use of an outbred population was that certification that any third party animal was genetically distant from mother or foetus was impossible. As a consequence, it was necessary to retain some reservations about the extent to which common alterations in reactivity against foetal (or maternal) and third party cells indicated the immunological non-specificity of those alterations.

Selection of the parameters to be used to detect and interpret pregnancy-associated changes in both expressed and potential reactivity was determined by availability of specimens, reproducibility of different assays and the likelihood that a phenomenon would be directly related to the course of the pregnancy. As regards availability of specimens, paternal cells were substituted for foetal cells on some occasions. The relative stimulatory

capacity for maternal lymphocytes of paternal and foetal cells could differ substantially if the foetus lacked a number of strong histocompatibility determinants expressed by the ram, or if foetal cells expressed unique differentiation antigens not retained by adult lymphocytes. However, the general patterns of reactivity of maternal lymphocytes against paternal and foetal cells did not differ significantly in the present experiments and, in those pregnancies in which both paternal and foetal efferent cells were tested as stimulators, responses to the two cell types were almost identical.

The reproducibility of assays in which the end-point is DNA synthesis in response to the exposure of lymphocytes to allogeneic cells or mitogens in vitro is not always adequate to detect trends that are not gross. This difficulty arises primarily because of variations in the basal response observed in batches of assays undertaken at different times. This difficulty was largely avoided in the present investigation by the regular use of cryopreservation to permit simultaneous assay of all samples of lymphocytes collected in any experiment.

There was little indication from the present experiments that significant changes in maternal immunological reactivity occur in (let alone exert an influence on the course of) normal pregnancy in the sheep. Concurrent examination of the response of maternal cells to foetal (or paternal) cells and to non-specific mitogens ensured that any changes in the former response were not masked because of compensatory variations in the 'base line' level of responsiveness in vitro. The only modification in maternal lymphocyte reactivity that requires comment was the frequent increase in responsiveness of maternal PBL to efferent lymphocytes (irrespective of source) and LPS in the later stages of pregnancy. The reason for the poor stimulatory capacity of efferent lymphocyte populations for normal PBL is unknown, although paucity of macrophages or of some lymphocyte subpopulation are possibilities. Whilst quite non-specific in a genetic sense, the pregnancy-induced augmentation of

lymphocyte responsiveness towards 'efferent lymphocytes', which resulted in their stimulatory capacity approaching that of peripheral blood cells, was sufficiently consistent to suggest that it could have some role in pregnancy. Possible mechanisms for this increased reactivity include an increased content of and LPS-responsive subclass of cells able to augment the reactivity of the remaining maternal cells, a general increase in the responsiveness of the entire maternal lymphocyte population without any alteration in its composition, and the presence of some augmentary humoral factor that was not removed by washing the cells. It would not be useful to speculate on the possibility that this increase in responsiveness has some equivalent in the intact ewe as the biological importance, if any, of the proliferative response that is measured in MLC is an open question. The conditions utilized in the MLC assay have been selected in its development in order to maximize the differences between experimental and control specimens rather than to increase the relevance of the findings to processes that occur in the intact sheep. The fact that the conditions which are empirically found to provide maximum stimulation for lymphocytes from pregnant animals do not necessarily coincide with the ideal conditions for cells from non-pregnant donors should draw attention to the arbitrary nature of the latter. The present observations of the modified responsiveness of pregnant ewes' lymphocytes to efferent lymphatic cells recalls reports of changes in optimal concentrations of mitogens for stimulation of cells from newborns (Carr, Stites and Fudenberg 1972) and pregnant women (Carr, Stites and Fudenberg 1973).

Attempts to relate the *in vitro* responses of cells from pregnant ewes to the behaviour of these cells in the donor animal examined the propositions that systemic humoral factors could modify responsiveness of the lymphocyte population as a whole and also that variation in lymphocyte reactivity in different anatomical locations might occur. To test the first possibility, the effects of maternal and foetal plasma and serum on responsiveness of lymphocytes from mother and foetus were examined. There were indications that

maternal plasma could depress lymphocyte reactivity. Whilst the possibility arises that this was an inhibitory effect of heparin, similar to that demonstrated by Currie (1967), the significantly greater depression produced by plasma during as compared with before pregnancy does not support this interpretation. However, the response of peripheral maternal lymph nodes to challenge with foetal lymphocytes did not give any indication that responsiveness of the ewe is impaired.

With regard to the possibility of variation between the reactivity of lymphocytes in differing anatomical locations, investigation was directed to the responsiveness of cells returning from the uterus. There have been a number of reports, conflicting in their implications, about the reactivity of cells from the para-aortic lymph nodes of pregnant mice (Harrison 1976, Bauminger and Peleg 1978, Head, Hamilton and Beer 1978, Gottesman and Stutman 1980). However, in the present study there was no consistent indication of any significant difference in reactivity between cells in the jugular vein and cells returning from the uterus.

The risk that specific modifications of the responsiveness of maternal lymphocytes may have escaped detection in the present experiments is small. The prominence with which secondary responsiveness was reflected in MLC kinetics ensured that spontaneous sensitization of maternal cells would not be overlooked, while the ready response of both mother and foetus to deliberate challenge with the cells of the other excluded the possibility of the spontaneous induction of an unresponsive state.

The possibility has been raised that proliferation of cells may not always correlate with increase in their reactivity (Nisbet and Simonsen 1967) and a contingent question was whether the nature of the cellular response in vivo was such as to be detectable by measurement of DNA synthesis of cells in vitro. In response to this possibility in vivo assays were used in conjunction with in vitro assays in the current study whenever possible.

The response of the pregnant ewe to deliberate challenge with foetal and

paternal cells closely resembled that of normal non-pregnant sheep. While the detailed mechanisms underlying the decrease in reactivity of the cells migrating from an antigenically challenged node remain unclear, this phenomenon is a very reproducible feature of the normal immune response. Consequently, had this feature been lacking in the maternal response to foetal cells, it would have suggested that some modification of maternal reactivity, albeit too subtle to be detected by the other assays, had occurred. The regular occurrence of the phenomenon strengthened the conclusion, already drawn, about the normality of maternal response.

The failure of any of the techniques employed to detect cytotoxic cells (despite the presence of cytotoxic antibody) in response to challenge of normal or pregnant sheep precluded the use of this type of assay for probing reactivity during pregnancy. However, given the dubious biological relevance of many cytotoxicity phenomena (McCullagh 1978), this was probably not a significant loss.

Efforts to induce immunological tolerance in young foetuses were hampered by inordinately high losses of inoculated animals. Given that transfer of fully (as distinct from semi) allogeneic haemopoietic cells to neonatal rodents is frequently followed by lethal runting, a similar occurrence is the most likely explanation for the present result. Nevertheless some tentative inferences might be formulated from consideration of the survivors. Clearly, there was a divergence between the results of different assays of immunological competence which seems to emphasize the inadequacy of our understanding of the meaning of these tests. The very weak NLT responses and strong serum inhibitory activity observed in some of the lambs that had been inoculated in utero do suggest that the reactivity of these animals had been modified by the earlier treatment. Unfortunately, the small number of survivors prevented any analysis of the relative efficacy of bone marrow versus lymph node cells of allogeneic versus maternal cells or of the effect of age of inoculation. The normal lymphocyte responsive-

observed in the case of the two lambs which were inoculated as young foetuses and submitted to thoracic duct cannulation later in gestation was not consistent with any proposition that some extent of tolerance had been induced but had been replaced by normal reactivity during maturation.

Examination of the response of the intact, older foetus to deliberate transfer of maternal lymphocytes gave little indication of any difference from the response to be anticipated on the part of an adult sheep challenged with allogeneic cells. The one aspect of this response in which foetuses seemed to differ from ewes was in the absence of cytotoxic antibody production. This failure is unlikely to be attributable to general immaturity of antibody production as foetuses of the age of those examined (from 110 to 150 days) have been shown to be competent to respond to other antigens (Silverstein and Kraner 1965, Silverstein and Prendergast 1970, Fahey 1976). It is possible that the absence of cytotoxic antibodies after challenge of the foetus with maternal lymphocytes may have resulted from a specific failure of response against these cells because of their origin. However, as it was not practicable to examine the response of a group of foetuses to lymphocytes from unrelated sheep, this possibility cannot be further explored.

Apart from the immunological response of the foetus against injected maternal cells, the other clear feature of this set of experiments was the catastrophic effect of transferring sensitized maternal cells to the foetus. The rapidity (12-24 hr) with which death ensued is certainly not consistent with any recognized form of graft-versus-host reaction but is much more suggestive of a response of acute anaphylactic type. While the lymph from which sensitized maternal lymphocytes had been harvested usually contained cytotoxic anti-foetal antibodies, preliminary washing should have effectively removed these before transfer. It is possible that the dramatic differences observed between the transfer of normal and sensitized maternal cells may have

been of a quantitative nature. For example, the outcome of transfer of allogeneic cells to the foetus may have depended upon the relative speed of initiation of immune responses by those cells and the recipients' lymphoid system.

The general implications of the present experiments for mechanisms of maternal/foetal homeostasis, at least in the sheep, may be succinctly summarized as follows. If it is accepted that reactivity of maternal lymphocytes towards paternal or foetal cells does not change during pregnancy, the contingent question is whether lack of sensitization reflects some active mechanism operative in the pregnant animal which specifically precludes sensitization or whether effective placental separation of the two circulating cell populations suffices as an explanation. In the absence of any demonstrable mechanisms of the former type, one would strongly favour placental separation of maternal and foetal immune systems as a major factor in maintenance of homeostasis. Additionally, the indications of foetal competence to respond to maternal lymphocytes suggest the existence of an additional mechanism to protect the lamb against transplacental passage of these cells.

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