STUDIES ON THE TRANSPLANTATION OF RABBIT YOLK SAC

A thesis submitted for the degree of

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by

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Prologue

In an outbred mating the conceptus inherits part of its transplantation antigen complement from the paternal gamete. The conceptus is therefore allogenic in relation to its host the mother and should be subject to an allograft rejection response. The way in which the conceptus escapes this response constitutes one of the immunological problems of pregnancy.

The conceptus is made up of the foetus surrounded by the foetal membranes. The foetus contains transplantation antigens and is immunogenic at an early stage in gestation. The arrangement of the foetal membranes varies in the different species of mammals. In many cases the only tissue which makes contact with the maternal tissues is trophoblast which in general does not undergo an allograft rejection response.

The rabbit, mouse and guinea pig are unusual in that during gestation a membrane - the visceral yolk sac - which is made up of only mesodermal and endodermal cells, is brought into direct apposition to the maternal tissues. This particular feature was used to study the general question of whether foetal membranes other than trophoblast which are apposed to maternal tissues suffer an allograft rejection response.

This study was designed to determine the immunogenicity of the visceral yolk sac and then to study the relationship between the yolk sac and the maternal tissues.

The study is a continuation of the work of T.A. Andrew (1971). He studied the mouse visceral yolk sac which comes to lie in direct apposition to the maternal tissues at 15 days. He made

grafts of excised visceral yolk sac into isogenic and allogenic hosts. His results indicated that the visceral yolk sac is destroyed by an allograft rejection response and that the membrane probably contains transplantation antigens as early as 10 days post coitum.

In the rabbit the visceral yolk sac lies apposed to the maternal tissues at a relatively earlier stage in gestation (13 days) and the arrangement of the foetal membranes is different.

The thesis is divided into two parts. In the first the relationship between the foetal membranes and the maternal tissues is examined both during development and in the definitive arrangement which exists from day 13 to the end of gestation. Scanning electron microscopy and light microscopy were used, but the former proved to be of little use. A few incidental findings have been included in the results.

The second part describes the results obtained when visceral yolk sac was excised from the conceptuses of New Zealand white rabbits and transplanted to an ectopic site in coloured Dutch rabbits. The discussion aims to bring together these two sets of findings and to provide a theory as to how the visceral yolk sac of the rabbit escapes rejection during gestation.

INTRODUCTION TO THE MORPHOLOGICAL STUDY

INTRODUCTION TO THE MORPHOLOGY OF DEVELOPING CONCEPTUS

There are several advantages in using the rabbit for this investigation:

1. The fact that coitus induces ovulation, which occurs 10 ± 2 hours post coitum (Gregory 1930, Lutwak-Mann 1971) and the age of the conceptus can therefore be clearly defined, the day of mating being counted as day 0.
2. Each pregnant doe provides a large number of conceptuses.
3. The relatively large conceptuses are easily dissected.
4. Pregnant rabbits are available at most times during the year. The gestation period of the rabbit is 30 - 32 days (Gregory 1930).

Development of the blastocyst prior to implantation

Fertilisation of the ovum occurs in the ampullae of the oviducts (Davies J. et al 1971). The blastocyst enters the uterus at 72 - 74 hours post coitum. (Boving 1956). At this stage it consists of an inner cell mass at the embryonic pole and a single layer of trophoblast in the extraembryonic region. The blastocyst is surrounded by the zona pellucida and an outer "mucin" coat. The latter is derived from secretions of the oviduct (Gregory 1930).

During the next 4 days the blastocyst lies free within the uterine cavity and before implantation, undergoes a rapid expansion. On day 5 the extraembryonic wall is bilaminar (Steer 1969) and is still covered by the zona pellucida and "mucin" coat. During day 6 the primitive streak (Lutwak-Mann 1971) and trophoblast knobs

(Boving 1962) form in the embryonic and extraembryonic regions respectively. The blastocysts are now evenly spaced in the uterus (Boving 1956).

The Rabbit Uterus

The rabbit uterus is bicornuate, the cervices open into a common vagina. The two horns lie in the free edge of the mesometrium. The border of the uterus adjacent to the mesometrium is named the mesometrial border and that opposite, the antimesometrial border.

In the mature non-pregnant uterus the endometrium is thrown up into a series of symmetrical pairs of longitudinal folds which have been named by Minot (1890). The largest of these are the placental folds which lie on the mesometrial wall and are separated by the deep interplacental fissure. Adjacent to the placental folds are the periplacental folds and lying on the antimesometrial wall are the small obplacental folds. Each fold is divided by transverse fissures into rectangular coussinets (cushions) (see Fig. 1 and 2) (Hollard 1863).

Microscopic details

The epithelium of the uterus consists of simple columnar cells bearing microvilli and occasional long kinocilia (Kanagawa et al 1972). Simple mucus secreting glands open into the fissures between the folds. A capillary plexus underlies the epithelium and is particularly well developed at the tips of the placental folds (Parry 1950). This reflects the functional significance of these folds which eventually form the maternal part of the

chorioallantoic placenta.

Changes in structure prior to implantation

During pregnancy, in the period prior to implantation there is an increase in the size and vascularity of the placental and to a lesser degree of the periplacental folds (Hafez and Tsutsumi 1966). On day 2 the uterine epithelium proliferates and there is a marked increase in the absolute number of uterine glands (Parry 1950). By day 6 the placental folds are grooved by the formation of branching folds of endometrium (Hafez and Tsutsumi 1966). With the rapid expansion of the blastocyst at day 5, 6 and 7 the antimesometrial wall of the uterus is stretched and the obplacental folds are obliterated.

The cytological changes occuring prior to implantation of the blastocyst are disputed. Larsen (1961) states that from the 4th day most of the uterine epithelium loses its cell boundaries and is transformed into a symplasma (a homogeneous mass of cytoplasm containing groups of small nuclei), but this view has been challenged by Boving (1962), Enders and Schlafke (1969) and Steer (1971) who all show that trophoblast knob formation occurs about 24 hours before symplasma formation which they say occurs early on the 7th day.

Implantation of the blastocyst

This is the time at which the conceptus makes contact with the maternal tissues and in fact, conceptal and maternal tissues are brought into continuity during implantation of the blastocyst. Theoretically an allograft rejection response should be generated at this time.

Implantation in the rabbit is central and superficial with strict orientation of the blastocyst so that the embryonic plate lies in apposition to the placental folds.

Boving (1962, 1971) has studied and reviewed the processes of implantation and orientation of the blastocyst.

Implantation is a continuing process and blastocysts in an individual animal are at different stages of implantation. Enders and Schlafke (1971) discerned 3 stages which form a chronological sequence.

1. Blastocysts held in position within the uterus.
2. Blastocysts with obvious sites of attachment antimesometrially.
3. Blastocysts with mesometrial and antimesometrial attachments.

Larsen (1961) states that mesometrial attachment occurs about 24 hours after antimesometrial attachment.

The sequence of events which occur during implantation, both at the mesometrial and antimesometrial poles can be divided into three stages:

1. The appositional stage with the blastocyst, surrounded by the zona pellucida, lying directly apposed to the epithelium of the endometrium in such a fashion that it will not be displaced during subsequent development.
2. The adhesive phase with the association of the blastocyst and uterine epithelium being so intimate as to prevent dislocation by physical means.
3. The invasive phase when there is penetration of the uterine epithelium.

Orientation of the blastocyst occurs during the first two

stages: the actual mechanism is disputed, Boving (1971) suggests a chemotactic theory whilst Assheton (1895) and Enders (1971) propose a purely physical basis in which differential compressibility of the embryonic cell mass may account for consistent orientation.

The invasive phase

Prior to the invasive phase the blastocyst is separated from the surrounding maternal tissues by the zona pellucide. In an outbred mating tissues of differing histocompatibility are brought into apposition when the maternal tissues are invaded by the conceptus. This first confrontation between maternal and foetal tissues is of interest from an immunological point of view and therefore will be described in some detail.

During the 6th day trophoblastic knobs appear on the extra-embryonic surface of the blastocyst (Fig. 1). (Enders and Schlafke 1969). A single knob consists of outer syncytiotrophoblast composed of fused ectodermal cells and lying deep in apposition to the basement lamina, there are several multinucleated cytotrophoblastic cells (Steer 1969). The invasive phase of implantation begins early on the 7th day and is completed by about 7 days 20 hours.

Steer (1970, 1971) points out that there are two mechanisms of trophoblast knob invasion and clarifies the confused picture that arose out of the work of Larsen (1963).

Trophoblast knobs type A occur along the abembryonic border of the blastocyst; type B knobs occur adjacent to the embryonic plate which lies in juxtaposition to the placental folds.

Type A invasion

The syncytiotrophoblast is insinuated as a wedge between the endometrial epithelial cells; only a single point of invasion is seen for any one knob although the syncytiotrophoblast overlaps several epithelial cells prior to insinuation (Enders 1971). The syncytiotrophoblast is joined to the adjacent maternal epithelial cells by zonae occludentes and small punctate desmosomes (Enders and Schlafke 1969). In places the cell membranes lose their identity and the two cells types appear to fuse; the cytoplasms of the apposed cells initially retain their individual characteristics and are separated by a dense ill defined zone (Steer 1971).

Thus cells of differing histocompatibility are brought into intimate contact.

Type B invasion

Before this type of invasion occurs the uterine epithelium is converted into a symplasma. The syncytiotrophoblast fuses directly with the surface of the symplasma and does not insinuate itself between the fused cells. The line of fusion is marked by a zone of large PAS positive vesicles (Larsen 1961). Even ciliated cells on the placental folds form part of the symplasma and fuse with the syncytiotrophoblast (Enders and Schlafke 1971).

By 7 days 18 hours the whole of the uterine epithelium is converted into a symplasma except for that epithelium which lies in the deepest recesses of the uterine glands (Sansom 1927).

The further development of implantation is the same in both type A and B invasion and will be described together [redacted]

The syncytiotrophoblast of the trophoblastic knobs is now completely fused with the uterine symplasma and as the invasive

[redacted] 4

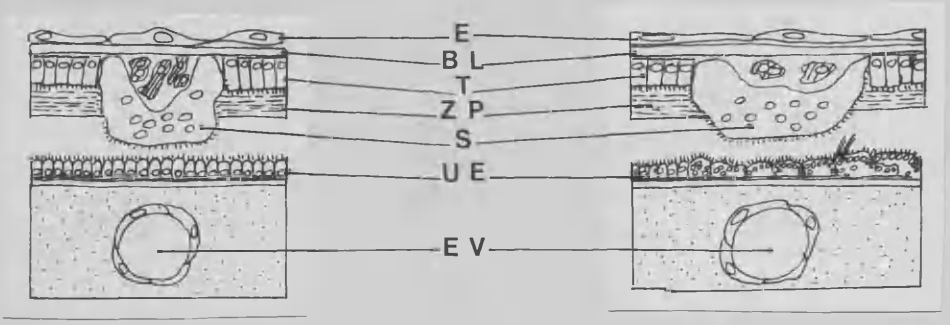
Trophoblast knob development and invasion of the maternal tissues (after Steer 1971) (see text for description)

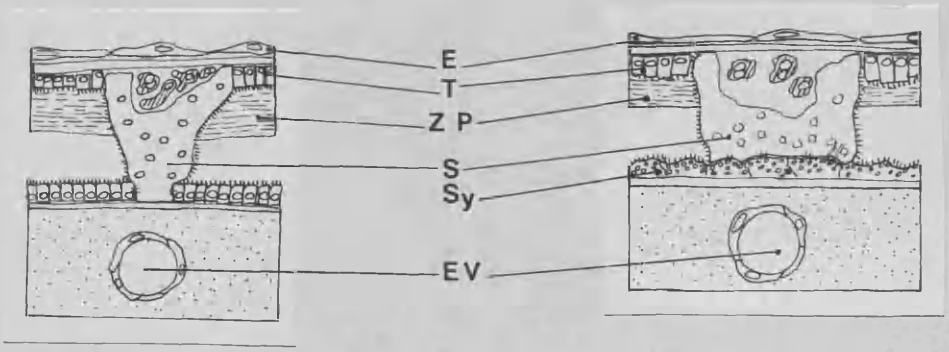
KEY

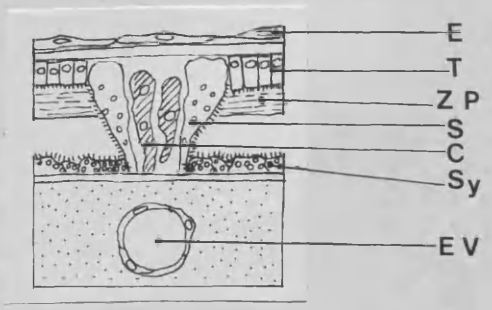
E	=	Endoderm	Bilaminar omphalopleu
BL	=	Basal lamina	
T	=	Trophoblast	
ZP	=	Zona pellucida	
C	=	Cytotrophoblast of knob	
S	=	Syncytiotrophoblast of knob	
UE	=	Uterine epithelium	
EV	=	Endometrial blood vessel	
Cy	=	Cytotrophoblast penetrating endometri vessel	
Sy	=	Symplasma	

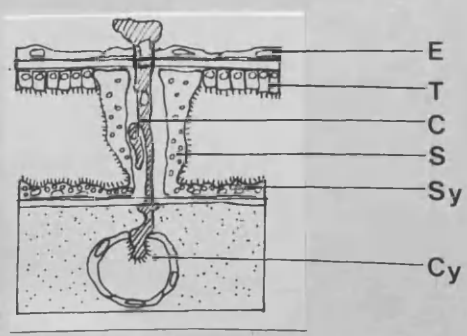
TYPE A

TYPE B









phase continues the line of demarcation between the two cell types is lost (Larsen 1963).

During this period of fusion the multinucleated cytotrophoblastic cells which lie deep to the syncytiotrophoblast perforate the basal lamina of the blastocyst wall and send central processes into the space between the basal lamina and the endoderm of the blastocyst. These same cells send out peripheral processes which push through the fused syncytiotrophoblast so that they abut against the basal lamina of the uterine epithelium. The two types of trophoblast are separated by an extracellular space, occasionally their membranes are joined by desmosomes. Thus the syncytiotrophoblast separates the invading cytotrophoblast from the uterine symplasma.

The cytotrophoblast now perforates the basal lamina of the uterine epithelium and spreads out deep to it. Cytoplasmic processes with a covering of microvilli, then insinuate themselves between the dilated pericytes and endothelium of an underlying maternal capillary passing directly into the blood vessel lumen. A fibrin and platelet clot forms over the invading tip. (Steer 1971b). By this time the endoderm of the blastocyst has been separated by the central processes of the cytotrophoblast. Thus there is only one cell layer separating the yolk sac cavity from the maternal uterine blood vessels. It is at this time ($7\frac{1}{2}$ days) that the maternal plasma proteins are first found in the yolk sac (Brambell and Hemmings 1949).

The origin and cytology of the obplacental giant cells

Following the invasive phase of implantation, giant cells are

seen in the antimesometrial wall of the uterus. Whilst examining the grafts of yolk sac (see results of grafting experiments) cells of similar appearance were seen. I have, therefore, described details of the obplacental giant cells in the following section in order to ascertain their origin.

The obplacental giant cells are seen in the antimesometrial uterine wall at 9 days (Sansom 1927) and are present throughout the latter half of the pregnancy. Initially they are found only in the submucosa (Larsen 1963). The giant cells are migratory (Sansom 1927) and from day 13 are found amongst the longitudinal and circular muscle fibres of the myometrium as well as in the submucosa. (Sansom 1927, Davies and Halmi 1953). They do not have a destructive influence on the surrounding maternal tissues (Sansom 1927, Larsen 1963).

At day 9 the cells are 20-30 μm in diameter; there is then an increase in the size and number of the cells reaching a maximum size of 100-200 μm on day 17 (Larsen 1963). After day 17 the cells decrease in size until at full term no giant cells are seen (Sansom 1927).

Most of the giant cells are mononuclear although multinucleated cells are occasionally seen. The cells are surrounded by a scalloped PAS positive border (Davies and Halmi 1953). On electron microscopy the cytoplasm contains a well developed golgi apparatus, many PAS positive cytoplasmic inclusions at the periphery and a complex infolding of the cell membrane (Larsen 1963). These features suggest that the cell is involved in secretory activity. The cells are sometimes seen in contact with endothelium, but they

do not line the interior of the vessel (Larsen 1963).

The origin of the giant cells is disputed and not yet resolved. Samsom (1927) believed that the majority are of foetal origin and states that most of them migrate to the obplacental wall from the free edge of the remaining bilaminar omphalopleure. Davies and Halmi (1953) believed that some of the cells are of myogenic origin and presented evidence by light microscopy, of the cytoplasm of both muscle cells and giant cells in continuity. Larsen (1963) disproved this theory, showing by electron microscopy that intact cell membranes separated the cells and goes on to state that the majority of cells originate from invading trophoblast.

It seems likely that the giant cells originate from the invading trophoblast knobs, but this idea remains speculative in the light of present evidence.

Development of the foetal membranes

From day 8 to day 12 the conceptus undergoes a series of changes which convert it from a spherical blastocyst to a complicated arrangement of membranes investing the foetus. A transverse section of a day 13 conceptus is shown in [illegible].

The bilaminar omphalopleure

At about day 7, mesoderm spreads from the embryonic region until it almost reaches the equator of the spherical blastocyst. The abembryonic hemisphere is therefore composed of only two cell layers - endoderm and trophoblastic ectoderm. This membrane is called the bilaminar omphalopleure, non-vascular yolk sac or somatic yolk sac. The non-vascular choriovitelline placenta (Amoroso 1952)

is established during the invasive phase of implantation. Degeneration of this membrane occurs on the 11th and 12th days (Larsen 1963, Brambell 1970). During the 9th and 10th days arcades of bilaminar omphalopleure form between the sites of trophoblast invasion (see Fig. 24). The symplasma-trophoblast complex degenerates, the arcades are freed and the complex sloughs into the uterine lumen. The denuded uterine surface is replaced by migration of cells from the deep parts of the glands in the antimesometrial wall. (Amoroso 1952). The whole of the bilaminar omphalopleure degenerates except for a circumferential fringe.

(see [illegible]). By day 13 the yolk sac cavity and the uterine lumen are one.

It is noteworthy that the endoderm of the bilaminar omphalopleure never makes direct contact with the maternal tissues; the only tissue which does so is of trophoblastic origin.

The visceral yolk sac

The development of the visceral yolk sac is not well documented. [illegible] are taken from histological sections at day 9 and day 10, and should be consulted in the following description. Brambell (1970) states that the exocoelom forms as the mesoderm spreads from the embryonic region. It is obvious from [illegible] that the exocoelom forms about 2 days after the mesoderm has spread.

The exocoelom forms by a separation of the mesoderm into a splanchopleuric layer which is associated with the endoderm of the visceral yolk sac and a somatopleuric layer which is associated with the ectoderm.

As the exocoelom forms, the visceral yolk sac is inverted into

the yolk sac cavity, thus bringing the endoderm of the vascular and non-vascular yolk sac into close proximity (Amoroso 1952).

Haemoipoietic islands develop in the splanchopleuric mesoderm very soon after its appearance. A large vessel, the sinus terminalis, demarcates the junction between the visceral yolk sac and the bilaminar omphalopleure. The vitelline circulation is established early on the 9th day (Luse 1958).

Davies and Hesseldahl (1971) state that inversion occurs on the 12th day around the axis of the sinus terminalis. This results in apposition of the visceral yolk sac and the bilaminar omphalopleure which according to the authors breaks down on the 17th day. In my preparations the bilaminar omphalopleure had completely broken down and the uterine epithelium regenerated by day 13. The endoderm of the visceral yolk sac and the uterine epithelium were therefore not separated by a membrane. All authors, Amoroso (1952), Larsen (1963), Davies and Hasseldahl (1971), are agreed that when the bilaminar omphalopleure breaks down, the visceral yolk sac comes into contact with the uterine epithelium. Larsen (1963) states that "the inverted yolk sac is kept in contact with the antimesometrial uterine epithelium by the pressure of fluid in the amniotic cavity and extraembryonic coelom". However, none of the authors present photographic evidence of direct contact; in all photomicrographs the visceral yolk sac is separated from the uterine epithelium by the contents of the uterine lumen. Moreover, Brambell (1970) states that "although the endoderm is pressed close to the regenerated uterine epithelium it neither fused with it nor is even in contact with it at any stage".

The relationship between the uterine epithelium and the visceral yolk sac

It is apparent that it is of paramount importance to know the precise relationship between the visceral yolk sac and the uterine epithelium. The details will be presented in the discussion.

Structure of the inverted yolk sac placenta

The inverted yolk sac placenta is established after breakdown of the bilaminar omphalopleure. The foetal part consists of the visceral yolk sac and the maternal part is represented by the uterine epithelium lining the antimesometrial wall of the uterus. At 18 days the latter is composed of tall columnar cells each with a single nucleus. The cell surface is covered by slender, regular microvilli and the cytoplasmic organelles are indicative of the secretory nature of these cells (Larsen 1963).

Morris (1950) has described the structure of the visceral yolk sac membrane at 16 and 24 days using light microscopy. The membrane is divisible into three parts; the outer endoderm, the intermediate loose mesenchyme and a mesothelial layer which faces the exocoelom.

The endoderm consists of columnar cells which hypertrophy during gestation. The luminal surface of the cells is covered by a brush border. Lying deep to the brush border is the apical cytoplasm are a large number of osmiophilic granules.

In the intermediate loose mesenchyme there are vitelline vessels which create ridges in both the overlying endoderm and the underlying mesothelial layer.

On electron microscopy the endodermal cells can be seen to rest on a basal lamina. The luminal surface is covered by irregular

microvilli (Larsen 1963) which at 26 - 28 days have a length of 0.5 - 0.7 μm (Slade 1969). At 26 - 28 days the columnar cells are 20 x 10 μm and rest on a basal lamina of 2 - 3 μm in thickness (Slade 1969). Large inclusions are seen in the apical cytoplasm at 14 days, some of these are fat droplets (Luse 1958). The nucleus is located near the base of the cell and contains one or two nucleoli; fat inclusions are occasionally seen within the nucleus (Larsen 1963). There are no large cytoplasmic inclusions by 26 - 28 days (Slade 1969). The basal plasmalemma is flat and follows the basal lamina perfectly.

The endodermal cells are absorptive in function. At first sight uptake appears to be non specific, the cells will absorb such diverse substances as cream, red blood cells, and gold particles (Luse 1957). However, Brambell (1971) and others have demonstrated that this membrane is involved in the transfer of passive immunity and that transmission of antibodies to the vitelline circulation is selective. The cell membranes of the endodermal cells are specialised for attachment of the Fc fragment of the immunoglobulin. The endodermal cells as therefore highly specialised and involved in specific and non specific absorption of materials from the uterine lumen.

The accessory foetal membranes

The paraplacental chorion

This membrane lies between the sinus terminalis and the mesometrial implantation site; it lies adjacent to the periplacental fold and within the circumplacental furrow.

Amoroso (1959) named the membrane; previous authors call it the chorion laeve. It consists of two intimate layers - trophoblast and splanchnopleuric mesoderm. The former is continuous with the

trophoblast of the remaining bilaminar omphalopleure.

The trophoblast layer remains relatively quiescent until the 14th day when multinucleated spherules bud off from it; maximum activity is attained on the 21st day and then wanes until parturition (Samson 1927). Some of these spherules invade the paraplacental folds resulting in breakdown of the decidua and epithelium of these folds. This debris sloughs into the uterine cavity carrying with it the invading spherules (Larsen 1962).

At about the 13th day the mesoderm of the paraplacental chorion is reinforced by spread of the allantoic mesoderm (Morris 1950). This part of the allantoic mesoderm splits into an outer avascular layer in contact with the established splanchnopleuric mesoderm and an inner vascular layer. Blood vessels in the latter anastomose with the sinus terminalis bringing the vitelline and allantoic circulations into communication (Duval 1892). The cavity produced on the 16th day by splitting of the allantoic mesoderm is called the accessory extraembryonic coelom (Brambell 1970).

The function of the paraplacental chorion is unknown, Samson (1927) suggests that the activity commencing on day 14 reduces the area of attachment of the placental disc preparatory to parturition. This seems a rather precocious preparation for parturition.

The allantois and formation of the chorioallantoic placenta

Attachment of the trophoblast overlying the placental folds occurs on the 8th day and involves a horse-shoe shaped area of uterine epithelium (Duval 1892).

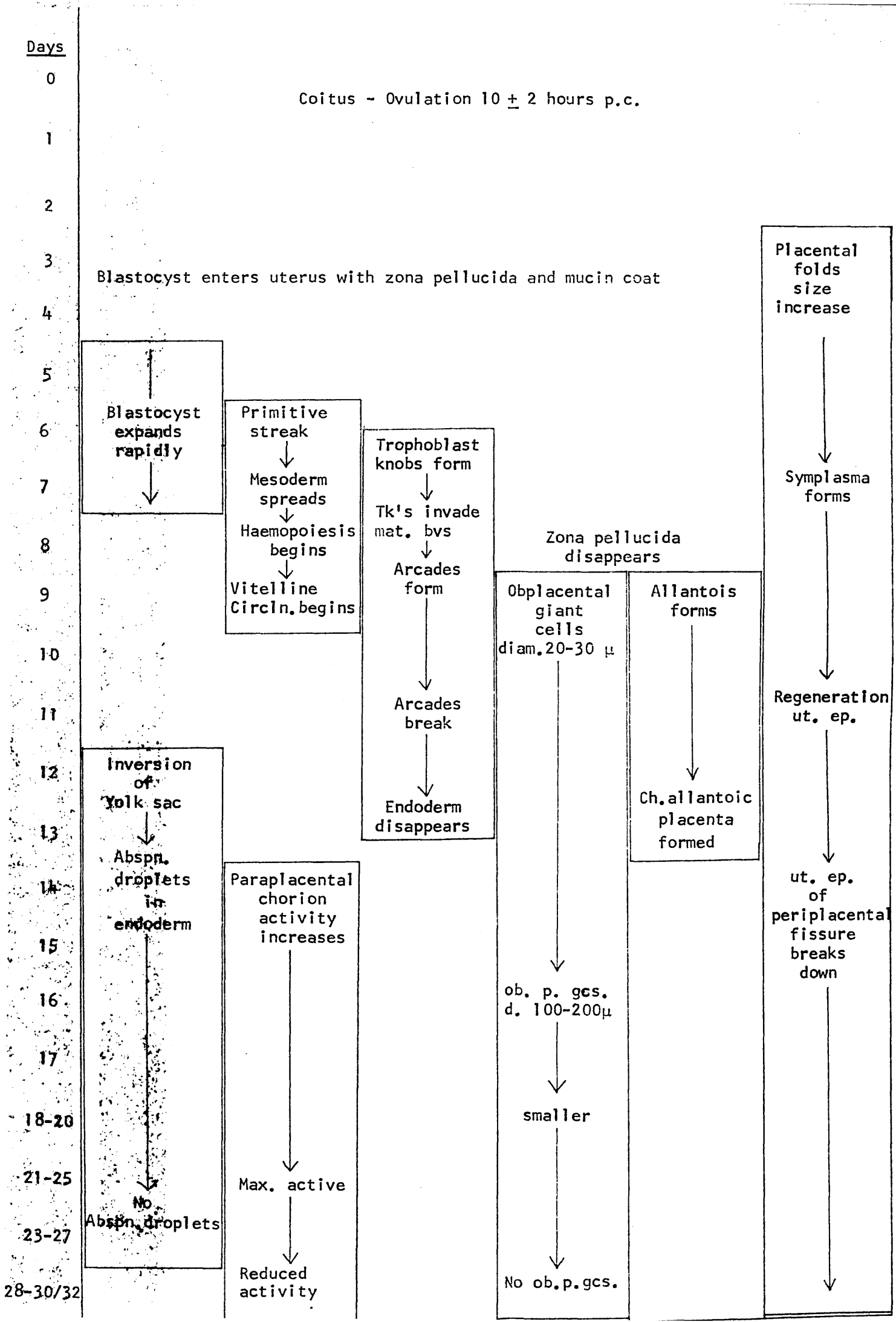
The grooves in the placental folds (Fig. 7) are filled with invading trophoblast by the end of day 8 (Hafez and Tsutsumi 1966).

The general structure of the placenta is established by day 14. It consists of a labyrinthine haemochorial arrangement (Larsen 1962b, 1963) with foetal and maternal blood flows in opposite directions (Mossman 1926). Thus the tissues which separate maternal and foetal blood are syncytiotrophoblast, cellular trophoblast, foetal mesenchyme and foetal endothelium.

The allantois has two components; an inner layer of endodermal epithelium, which arises as an outgrowth of the hindgut on the 9th day (Amoroso 1952) and an outer layer of vascular medoderm which finally links the foetus with the avascular chorion to form vascular allantochorion, the foetal component of the chorioallantoic placenta.

The amnion

Amniotic folds appear late on day 8 and fuse at the end of day 9 (Davies 1957). The amnion consists of an inner layer of squamous ectoderm resting on a basal lamina, external to which is an intermediate layer of connective tissue mesenchyme with collagen bundles and an outer discontinuous layer of mesenchyme. The membrane is separated from all other foetal membranes by the exocoelom (Larsen 1963).


Days
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18-20
21-25
23-27
28-30/32
Coitus - Ovulation 10 ± 2 hours p.c.
Blastocyst enters uterus with zona pellucida and mucin coat
Blastocyst expands rapidly
Primitive streak
Mesoderm spreads
Haemopoiesis begins
Vitelline Circln. begins
Trophoblast knobs form
Tk's invade mat. bvs
Arcades form
Arcades break
Endoderm disappears
Zona pellucida disappears
Obplacental giant cells diam. 20-30 µ
ob. p. gcs. d. 100-200µ
smaller
No ob.p.gcs.
Allantois forms
Ch.allantoic placenta formed
Placental folds size increase
Symplasma forms
Regeneration ut. ep.
ut. ep. of periplacental fissure breaks down
Inversion of Yolk sac
Absptn. droplets in endoderm
No Absptn. droplets
Paraplacental chorion activity increases
Max. active
Reduced activity

INTRODUCTION TO THE FOETUS

AS AN ALLOGRAFT

CELL MEDIATED IMMUNITY

Immunity is a state of heightened responsiveness such that antigen is bound or eliminated more rapidly than in the non immune state. (Herbert and Wilkinson 1973).

Lymphocytes are the cellular basis of immunity and are divided morphologically into two groups: small lymphocytes 5 - 8 μ diameter, medium sized lymphocytes 8 - 12 μm diameter (Weiss 1972). Morphologically they appear as a homogeneous collection of cells, but on a functional basis they can be divided into two broad populations; thymus derived lymphocytes (T lymphocytes) and a bursa derived lymphocytes (B lymphocytes). (Roitt et al 1969).

In the chicken, competent lymphocytes originate from the thymus and from the bursa of fabricius (Cooper 1965, Cooper 1966). Lymphocytes originating from the latter are involved in immunoglobulin production (Cooper et al 1969). The mammalian equivalent of the bursa has not yet been discovered, but it is evident that the population of the mammalian lymphocytes, known as B lymphocytes, is involved in immunoglobulin production. T lymphocytes mediate delayed hypersensitivity and transplantation immunity, act as killer cells in these two responses and have a helper effect in antibody production by B lymphocytes (Miller 1972).

The remainder of this introduction will be restricted to the relevance of T lymphocytes in the allograft response.

The origin of T lymphocytes

The unitarian theory is now generally accepted. This encompasses the concept that a single stem cell is the primary source of all blood cells (Maximow 1924). The stem cell has been named

the haematocytoblast (Maximow 1924) or large basophilic cell (J.J. Owen 1970).

Early in the development of all vertebrates, islands of haemopoietic cells develop in the yolk sac mesoderm. These contain the stem cells which give origin to the myeloid and lymphoid series (Wu 1968).

The stem cells are seeded to the various organs which undertake haemopoiesis and lymphocyte maturation during late foetal and adult life (Moore and Metcalf 1970). During foetal life in the chicken and other vertebrates the lymphoid population of the thymus is derived from stem cells which enter the epithelial primodium from the blood stream (Moore and Owen 1967). These large basophilic cells enter the thymic cortex and undergo proliferation, giving rise to a population of medium and small thymocytes which bear high concentrations of surface foetal and TL isoantigens (Owen 1971). The small thymocytes migrate to the thymic medulla and some enter the blood circulation. They have the same characteristics as T lymphocytes removed from the thoracic duct and are probably one and the same cell type. They are seeded to the thymus dependant areas of lymphoid tissue (Parrott et al 1966),(Goldschneider and McGregor 1968) and become part of the circulating pool of small lymphocytes.

The recirculation of small lymphocytes

Small lymphocytes circulate between the blood and the lymph (Gowans 1959). The small lymphocytes enter the mid and deep cortex of the lymph nodes from the post capillary venules (Gowans and Knight 1964, Goldschneider and McGregor 1968) by passing through

the cytoplasm of the cuboidal epithelium which lines these vessels (Marchesi and Gowans 1964). The lymphocytes come to lie in the T dependant area of the lymph node. It would therefore be expected that most of the circulating lymphocytes are T lymphocytes and indeed 80 - 90% of thoracic duct small lymphocytes carry the foetal isoantigen (Miller and Sprent 1971). The circulating small lymphocytes leave the lymph node cortex and pass in the efferent lymph to the thoracic duct. The characteristics of small lymphocytes in the thoracic duct have been studied in detail. The cells recirculate many times during their lifetime (Gowans 1959) with an average lifespan of eighteen months in the rat, although in humans some lymphocytes are still present after several years (Everett et al 1964). Recirculation of small lymphocytes occurs through all lymphoid tissue except the thymus (Gowans and Knight 1964).

The allograft rejection response

The response is elicited by transplanting suitable tissue to a suitable site between two genetically dissimilar mature members of the same species at certain graft dose levels. The result of such a transplantation is death of the donor graft.

This response satisfies the criteria for its inclusion under the definition of immunity. Thus a second set phenomenon occurs after sensitising the recipient and then applying a second graft which carries the same transplantation antigens as the sensitising graft (Medawar 1944). The sensitising effect is dose related and is systemic rather than local (Gibson and Medawar 1943, Medawar 1944, 1945).

Evidence for the involvement of T lymphocytes in the allograft response

Chronic thoracic duct drainage causes a depletion of small lymphocytes and an increased survival time of skin allografts (McGregor and Gowans 1964). Thus it would be expected that T lymphocytes are actively involved in the allograft response and indeed neonatal thymectomy of mice (Miller 1961) and rats (Arnason 1962) causes a severe depletion of circulating T lymphocytes and a prolonged survival of skin allografts.

The allograft response

The mechanism by which rejection occurs differs according to the tissue grafted. A brief summary will be presented and this will allow a classification to be made of the ways by which the foetus may escape rejection.

The mechanism has been arbitrarily divided into an afferent limb, a central response and an efferent or effector limb.

Afferent limb

The initial stimulation of the mechanism requires a difference between the transplantation antigen complement of donor and host.

T antigens are located on the cell membrane (Natheson 1970). Therefore the basic prerequisite is that competent T lymphocytes make contact with the T antigens of the graft or with a product resulting from recognition by the host that there is histo-incompatibility between itself and the graft. It has not yet been resolved where this confrontation occurs nor the mechanism by which it occurs.

The mechanism appears to be different for different tissues transplanted. Thus the median survival time of high dose skin allografts can be prolonged by transplanting the allograft to an

alymphatic pedicle of host skin (an artificial privileged site) (Barker and Billingham 1968). However, rejection does eventually occur, 200 - 300 days after grafting, sensitisation presumably occurring by the vascular route (Tilney and Gowans 1971). On the other hand kidney allografts experimentally deprived of their lymphatic drainage, sensitise the host 5 - 12 hours after establishing vascular anastomosis (Hume 1955, Strober and Gowans 1965).

In summary, sensitisation of the host can occur by the vascular route but with transplantation of certain tissues (e.g. skin), an intact efferent lymphatic drainage is required for sensitisation to occur within the normal rejection time for that tissue.

The site of confrontation between graft and host may also vary, for example in renal allografts, which have a rich vascular system sensitisation probably occurs at the surface of the glomerular capillary endothelium (Pederson and Morris 1970). In those grafts which depend on an intact lymphatic drainage for "normal" rejection to occur, for example skin, confrontation may occur at one of two sites:-

(a) Host lymphocytes pass into the graft where they become sensitised and then leave the graft by the afferent lymphatics draining the graft and pass to the regional lymph nodes (Medawar 1952, Hall 1969).

(b) Graft cells or debris pass in the afferent lymphatics draining the graft to the regional lymph nodes where a reaction occurs leading to sensitisation of the host.

Central response

The antigenic stimulus of skin allografts is brought to the

first regional lymph node. This is marked by an increase in the weight of the node and by the appearance of large pyroninophilic cells in the mid and deep cortex. This response is fully developed four days after the application of a skin graft (Scothorne and McGregor 1955). The cells are 12 - 15 μm in diameter with a thin rim of moderately basophilic cytoplasm (Andre et al 1962) which contains many free ribosomes (Gowans and McGregor 1965). These cells which are probably transformed T lymphocytes divide to produce a clone of lymphocytes of progressively decreasing size (Gowans 1966).

The sensitised lymphocytes now move out of the node in the efferent lymph (Hall 1967).

Efferent limb

The cellular infiltrate of an allograft is marked by the presence of large numbers of small lymphocytes, macrophages and occasional eosinophils. (Gibson and Medawar 1943). Some of the small lymphocytes are cytotoxic and originate from the original clone.

The process of graft cell destruction requires cell to cell membrane contact; thus a graft is not destroyed if it is isolated from the host by a millipore chamber which prevents the passage of small lymphocytes or other cytotoxic cells (Algire et al 1954). Also it has been observed that destruction is absent in those parts of epidermal grafts devoid of invading small lymphocytes (Wiener et al 1964). The number of infiltrating cells rises rapidly on the fourth day and reaches a maximum five days after grafting when graft rejection begins (Jakobisiak 1971). The median survival

time of high dose skin allografts is $10.4^{\pm}$ 1.1 days (Medawar 1945).

The mechanism of target cell destruction is unknown. In certain in vitro experiments, cultured cells are rapidly destroyed by lymphocytes without the presence of complement or humoral antibodies (Wilson D.B. 1963). Wiener (1964) noted that apposed cell membranes of target cells and lymphocytes are discontinuous in parts. It has recently been suggested that as the lymphocyte moves away from the target cell, it is able to repair its own membrane but leaves the target cell unable to repair its membrane resulting in lysis of this cell (Nature 1973).

The roles of the macrophage and eosinophil in the allograft response are largely unknown.

THE FOETUS AS AN ALLOGRAFT

Histocompatibility genes are accepted as being codominant. Thus the offspring of a mating involving two animals differing in their T antigen complement will express the whole inherited T antigen complement from both parents without dominance or interactions (Lengerova 1969). Theoretically the conceptus should behave as an allograft during its sojourn within the mother. Several reviews have been published concerning this problem of how the foetus survives within this alien environment (Medawar 1953, Billingham 1964, Behrman and Koren 1968, Kirby 1968, Beer and Billingham 1971).

It will become evident in the following introduction that no single factor can satisfactorily explain the exemption of the foetus from rejection. At present it seems that different mechanisms of protection may operate at different stages of pregnancy. It is also important to realise that the mechanisms may differ in different species and that a particular mechanism may be developed to a greater degree in a particular species.

I shall review firstly the evidence indicating that the conceptus contains transplantation antigens and secondly the possible sites of interference in the allograft response.

Conceptus antigenicity

1. THE FOETUS

Woodruff (1957) allografted 16 day foetal rabbit hind limbs into the flanks of presensitised recipients. These grafts were destroyed by an accelerated allograft response within six days.

At an earlier gestation period Simmons and Russell (1962, 1963) have shown that 7 day mouse embryos are destroyed by an allograft response. Schlesinger (1964) showed isoantigenic activity present in $10\frac{1}{2}$ day mice and a rapid increase of such activity between $13\frac{1}{2}$ and $15\frac{1}{2}$ days in the mouse liver.

The evidence for the presence of T antigens at earlier gestational periods is controversial. The results of Olds (1968) and Simmons and Russell (1966) suggested the presence of H-2 antigens at the two cell stage. However, Heyner and Brinister (1969) were unable to show H-2 antigens at the 8 cell stage using an anti-H-2 serum. Searle et al (1974) recently reported absence of H-2 antigens before the formation of the $7\frac{1}{2}$ day conceptus and these results support the findings of Palm et al (1971) who were only able to demonstrate H-3 and H-6 activity at the 2 cell and blastocyst stages using an antisera method.

In summary therefore, strong transplantation antigens cannot be detected by present techniques at the earliest stages of gestation; however in the mouse,T antigens do appear at $7\frac{1}{2}$ days and should theoretically promote an allograft response from the mother.

2. THE TROPHOBLAST

This tissue, which is present at the maternal-foetal junction of all viviparous species so far studied, (Wynn 1971), may act as a barrier between the antigenic foetus and the potentially reactive mother. The blood flow over this area is very high in haemochorial placentae. It is therefore tempting to compare the situation to that of the kidney allograft, i.e. that sensitisation may occur by

the vascular route rather than the lymphatic route.

Simmons and Russell (1962, 1963) transplanted $6\frac{1}{2}$ - $7\frac{1}{2}$ day old mouse conceptuses under the kidney capsule of an allogenic host. The trophoblast survived whilst the rest of the conceptus was destroyed within eleven days of transplantation. They also showed that transplantation of the ectoplacental cone of 7 day mice resulted in survival of trophoblast even in the presence of prior sensitisation.

Kirby et al (1966) showed that $3\frac{1}{2}$ day mouse blastocysts are destroyed totally when transplanted under the kidney capsule of an hyperimmune allogenic host. Simmons and Russell (1966) demonstrated total destruction of C3H eggs when transplanted to sensitised C57 recipients.

Currie et al (1968) treated mouse ectoplacental cone cells with neuraminidase and made an allogenic transplant of this tissue. This resulted in rejection of these trophoblastic cells.

Recently Jenkinson and Billington (1974), using in vitro methods, have shown that treatment of ectoplacental cone from $7\frac{1}{2}$ day mouse conceptuses with low concentrations of neuraminidase removes the mucoprotein cost. However, these cells are not destroyed by alloimmune spleen cells. They propose that some other mechanism inherent in the membrane of trophoblast may allow survival of these cells on transplantation. They also question the presence of T antigens on the trophoblast cell membrane.

In summary, therefore, the presence of T antigens on the trophoblast remains unanswered. The latest results mentioned above do not rule out the possibility that the mucoprotein layer

may act as a barrier to T antigen recognition. For example trophoblast may be able to resist the lytic power of the spleen cells and yet still contain T antigens.

3. OTHER FOETAL MEMBRANES

No work has been performed on the antigenicity of the amnion. The visceral yolk sac has been studied by Andrew (1971) and Andrew et al (1975) in the mouse and a preliminary study was made in the guinea pig. These findings indicated that the visceral yolk sac of the mouse contains transplantation antigens. He made no conclusions about the guinea pig yolk sac.

Mechanisms involved in protection of the foetus

I have divided this section into the three divisions of the allograft rejection response as previously described.

1. AFFERENT LIMB

The zona pellucida

This mucoprotein membrane which surrounds the blastocyst may act as a barrier in the early stages of gestation.

Heyner and Brinster (1969) found that removal of the zona pellucida led to a more rapid destruction of fertilised mouse eggs in the presence of antisera to T antigens. Searle et al (1974) found that removal of the zona pellucida led to a rapid destruction of ectopically allografted blastocysts. However Kirby (1969) found that prolongation of the zona free period by steroid administration during a normal pregnancy caused no difference in the subsequent pregnancy.

Simmons and Russell (1966) have suggested that the zona pellucida

allows the trophoblast to form in the developing conceptus unaffected by the maternal allograft response and that once the zona is digested (rabbit $7\frac{1}{2}$ days) the trophoblast takes over the role of protecting the foetus from rejection. Obviously Kirby's (1969) findings do not invalidate this hypothesis.

The uterus as a privileged site

The uterus may act as a privileged site during the early stages of pregnancy and may be especially important prior to the formation of the trophoblast barrier.

Schlesinger (1962) transplanted tumours into the endometrium of rodents and found that they were rejected; however the tumours outgrew the confines of the endometrium so the value of this experiment has been questioned. Poppa et al (1964) using parathyroid allografts implanted in endometrium found that rejection occurred at about 20 days; however these grafts did not stimulate the decidual reaction.

McLean and Scothorne (1970) were unable to demonstrate the presence of lymphatics in the endometrium of rabbits. The same authors (1971) found that epidermal allografts made to the endometrium had a prolonged survival when compared to similar grafts made to the uterine serosa which does contain lymphatics.

Decidual protection

Transfer of blastocysts beneath the kidney capsule of an allogeneic host results in an allograft rejection. However, if the blastocysts are transferred to the uterus of a pseudopregnant animal they will survive (Kirby et al 1966).

Trophoblast mucoprotein layer

The presence of a sialomucin layer may prevent the egress or hide transplantation antigens present on the trophoblast surface. Kirby et al (1964) showed an electron dense amorphous fibrinoid layer surrounding each trophoblast cell of the ecto-placental cone of the mouse conceptus. This layer is a mucoprotein rich in tryptophan and contains at least two non-sulphated acid mucopolysaccharides, possibly hyaluronic acid and sialic acid (Bradbury et al 1965). Currie (1967) suggested that the sialic acid residues contain a high negative charge which causes repulsion of lymphocytes. It is now generally considered that this is unlikely and that steric hindrance is a more probable mechanism (Currie 1968). Removal of this layer results in rejection of ectoplacental cone transplants (Currie et al 1968).

However, Potts (1965) noted that the sialomucin layer is absent when maternal and foetal mouse tissues make contact after rupture of the zona pellucida. Also workers have been unable to find a continuous fibrinoid layer in certain other species.

2. THE CENTRAL RESPONSE

It has been suggested that the central response may be diminished either specifically for paternal T antigens or non specifically for the allograft response in general. With the latter in mind several authors have suggested that the increased secretion of steroids during pregnancy may abrogate the allograft response on a non specific basis.

Heslop et al (1954) found that rabbit epidermal allografts survive about twice the normal rejection period between the 20th

and 24th day of pregnancy. This is supported by the finding that skin allografts made to the pregnant human survive for up to 18 days (Andresen 1962).

However high doses of oestradiol in mice (Medawar and Sparrow 1956), high doses of H.C.G. in mice (Pearse and Karman 1967) and high doses of progesterone (Simmons et al 1968) do not extend epidermal graft survival times.

Although at first sight steroids even at high doses do not appear to exert a significant systemic extension of graft survival time, it has been suggested that a high concentration of steroids at the trophoblast endometrial interface may diminish the allograft response locally. But, as Kirby (1966) points out, in these experiments in which trophoblast lives, proliferates and presumably secrete hormones the foetal tissue is destroyed by an allograft response unaffected by the supposed high local concentration of steroid hormones.

Hypersensitivity phenomena which are mediated by the T cell population are not diminished in pregnancy (Montgomery 1968). Thus the results of Heslop et al and Andresen have not been satisfactorily accounted for by this approach.

3. EFFERENT LIMB

As yet little is known about the efferent limb of the allograft response. It is noteworthy that pregnancy will take place in female mice made hyperimmune to the paternal T antigens, therefore the efferent limb must be affected in some way.

The resistance of female mice to a transplantable allogeneic tumour falls as a result of parity by a male mouse genetically

identical to the transplanted tumour (Breyere and Barrett 1960, Breyere 1961, Currie 1969). These authors suggest that enhancing antibodies produced by the mother to the paternal antigen component of the trophoblast or foetus coat the foreign T antigens and prevent cytotoxic lymphocytes from destroying the conceptus. However Kaliss and Dagg (1964) were unable to transfer enhancement by serum transfusions. Currie (1969) found that he was able to produce a specific unresponsiveness to paternal antigens by serum transfer from mice at day 18 of pregnancy and other results (Youtananukom et al 1972) suggest the presence of a plasma factor in post partum females which blocks the in vitro expression of the maternal cell mediated reaction to paternal antigens.

In summary no single mechanism can satisfactorily explain the protection of the foetus. Since the conceptus does not normally sensitise the mother during a pregnancy it seems that the afferent limb of the allograft response is blocked. However the conceptus does seem to have secondary forms of protection since pregnancy can occur in mothers presensitised to the paternal transplantation antigen complement (Beer and Billingham 1974). Thus the efferent limb or central response is also blocked.

MATERIAL & METHODS

FOR THE MORPHOLOGICAL STUDY

Materials and methods for the morphological study

Six mature female New Zealand white rabbits were used in the study. The rabbits were mated at a known time and then sacrificed on days 2, 7, 9, 10, 13 using an intravenous dose of Nembutal. (The day of coitus was taken as day 0). A non-pregnant uterus was also removed for study.

Fixation techniques

Non-pregnant uterus

The horns were dissected free of each other. A small section was removed from the middle of one horn and fixed in Bouin's solution. The other horn was opened along the antimesometrial border, pinned on to cork and not fixed.

2 day pregnant uterus

As this material was to be viewed on the scanning electron microscope (SEM) fixation was initiated as soon as possible.

The abdominal cavity of the rabbit was opened, a hole made in the anterior vaginal wall and a pipette containing glutaraldehyde 5% in phosphate buffer was inserted through the cervical canal of one horn. About 1 ml. of glutaraldehyde was introduced into the uterine cavity, care being taken not to distend the uterus. It was found unnecessary to ligate the oviduct or cervix to prevent escape of the fixative.

The horn was then dissected free and immersed whole in glutaraldehyde. After 3 hours the uterus was opened along the mesometrial border, pinned onto cork and fixed for a further 9 hours. A small block of tissue was removed from the centre and processed for

viewing by SEM.

This technique retains the natural fold pattern of the endometrium which would be distorted by immediate opening and pinning out of the uterus.

Techniques used at different gestation periods

Gestation Period (Days)	Tissue Studied	Technique of examination
0	Uterus	Gross, LM
2	Uterus	SEM
7	Uterus	SEM, LM
	Conceptus	LM
9	Uterus	SEM, LM
	Conceptus	LM
10	Uterus	SEM, LM
	Conceptus	LM
13	Uterus	SEM, LM
	Conceptus	LM
	Yolk Sac	SEM, LM, TEM

Gross = naked eye inspection

LM = light microscopy

SEM = scanning electron microscopy

TEM = transmission electron microscopy

7 day pregnant uterus

The uterine horns were removed, and one horn was placed in Bouin's solution. The other was fixed in glutaraldehyde 5% in phosphate buffer for 3 hours, when the implantation sites were dissected free of each other and opened along either the mesometrial or the antimesometrial border. The blastocyst was punctured and removed. The implantation sites were pinned on to cork and fixed for a further 9 hours. In those sites opened along the mesometrial border the antimesometrial wall was not visible, it was therefore inverted so that it stood up as a dome from the surrounding uterine folds. This method produces less distortion than stretching the uterus.

9 and 10 day pregnant uterus

Each site was separated by ligatures and fixed whole in Bouin's solution.

13 day pregnant uterus

The individual conceptuses were separated by ligatures and either fixed whole in Bouin's solution or dissected and fixed.

Processing of materials

Light microscopy -

All tissues were fixed in Bouin's solution, dehydrated, cleared and embedded in paraffin wax. Sections were cut at 3 - 5 μm thickness and stained using Haematoxylin and Eosin, periodic acid schiff reagent and Methylene Blue.

Scanning electron microscopy -

Tissues were fixed in glutaraldehyde 5% in phosphate buffer

at pH 7.4. They were then dehydrated through a graded series of alcohols and either ether/air dried or critical point dried.

In the former the tissues were soaked in a 50% ether/50% alcohol mixture for $\frac{1}{2}$ - 2 hours and then in 100% ether for $\frac{1}{2}$ - 2 hours. They were then air dried at room temperature underneath a glass cover.

In the latter the tissue was soaked in 50% amyl acetate/50% absolute alcohol mixture for $\frac{1}{2}$ - 2 hours and then in 100% amyl acetate for 1 hour. The tissues were then transferred to baskets and placed in the chamber of the critical point drier which contains 100% amyl acetate. This is replaced by flushing the chamber with liquid carbon dioxide and soaking the tissues for 3 - 4 hours. The pressure and temperature are adjusted until the liquid carbon dioxide reaches its critical point. The gaseous carbon dioxide is then released very slowly and the dry specimens removed from the chamber.

All specimens were mounted on stubs, coated with gold/palladium in a rotatory coater and viewed on the S600 Stereoscan microscope.

Note:- (1) It is important that all epithelial surfaces are free of debris and secretions. It is therefore mandatory to irrigate the surface with a stream of phosphate buffer prior to and after fixation.

(2) It is important not to stretch the material before fixation - this will destroy natural fold patterns and produce distortion of cells and other surface structures.

Transmission electron microscopy

After fixation for about half an hour in glutaraldehyde 5% in

phosphate buffer at pH 7.4, the tissue was placed in 5% osmic acid for half an hour, washed in phosphate buffer, dehydrated through a graded series of alcohols and using intermediate dilutions of propylene oxide finally embedded in araldite.

Ultrathin sections were cut using glass knives and the sections viewed through the Philips 200 Transmission Electron Microscope.

THE MATERIALS AND METHODS FOR THE GRAFTING STUDY OF YOLK SAC

The materials and methods for grafting experiments

Five adult female New Zealand white rabbits were used as donors and sixteen male coloured Dutch rabbits as recipients of the grafts. New Zealand white rabbits were mated and sacrificed at 13 - 13½ days.

Transplantation of the visceral yolk sac

The procedure was split into three parts. Instruments were sterilised in three separate trays and an individual tray was removed from the autoclave only when it was required. Animals were sacrificed and shaved in a separate room before beginning the procedure. A strict aseptic technique was adopted throughout.

1. REMOVAL OF THE UTERUS

A lower mid-line incision was made and the peritoneal cavity opened. The pregnant uteri usually lie superficially and appear as dark purple swellings. The uterus, oviducts and part of the vagina were delivered on to sterile gauze swabs and excised by cutting the mesometrium and vagina. These viscera were then laid in a sterile petri dish and covered by a saline soaked gauze swab. The New Zealand white rabbit donor was removed from the table.

2. REMOVAL OF THE YOLK SAC

The individual conceptuses were separated using mosquito forceps and scissors to clamp and cut the interconceptal uterus.

A single conceptus was laid on a sterile gauze swab such that the antimesometrial wall faced the operator. The forceps helped stabilise the conceptus during dissection and prevent herniation of the yolk sac through the uterine lumen.

The antimesometrial wall was picked up using fine tooth forceps and a cruciate cut made using ophthalmic scissors. The latter operation must be performed quickly and accurately, or else herniation and rupture of the yolk sac will occur making the rest of the procedure difficult.

The yolk sac is now displayed and the vitelline vessels are seen coursing over its surface. The foetal heart was observed to be beating even three quarters of an hour after sacrificing the mother. A thin layer of mucus covers the endoderm. This was removed using a pair of fine toothed forceps.

A puncture was made in the yolk sac just lateral to the vitelline vessels as they leave the yolk sac and enter the foetus. Ophthalmic scissors were then introduced into the exocoelom and the vessels transsected, thus separating the visceral yolk sac from the foetus. The yolk sac was then removed whole by making a circumferential cut just medial to the sinus terminalis. Occasionally part of the paraplacental chorion was removed with the yolk sac.

3. GRAFTING OF THE YOLK SAC

Coloured Dutch rabbits were weighed and anaesthetised using Nembutal (Veterinary - Abbott) 60 mg/cc as a stat. intravenous injection. The dose was about 0.66 mg per kilogram bodyweight and was injected very slowly into the marginal ear vein until the animal just responded to squeezing the ear. The dorsum of both ears were then shaved and soaked with hibitane swabs.

The Argyle Medicut intravenous cannula (Sherwood Medical Industries Incorporated U.S.) size 18 Ga x 2 inches was used for introducing the graft. A 2 ml syringe was filled with T199 and

attached to the needle. Avoiding all large blood vessels, the needle and cannula were introduced about 1 cm into the dermis. A small blister was then raised by injecting T199. This should cause a peau d'orange appearance of the overlying epidermis; if it does not, then the needle is lying in the wrong tissue plane and should be removed and another site selected.

The needle and syringe are then removed leaving the cannula in situ. The yolk wac was cut into small pieces and introduced into the bore of the cannula. The syringe was then replaced and the graft introduced into the blister by applying gentle pressure on the syringe. The cannula is then removed and the exit hole sealed immediately by pressure, thereby avoiding reflux of the graft. The exit hole was marked by a 6/0 silk suture and sprayed with antibiotic powder and Nebecutaine dressing.

About $\frac{1}{2}$ to $\frac{3}{4}$ of a yolk sac was introduced into the right and left ear of each rabbit.

Excision of the grafts

These were removed either immediately or at six, nine or fourteen days after grafting (see table). The graft was always evident as a small (c.2 mm square) lump and could therefore be accurately excised by cutting a full thickness window from the ear. The grafts were stitched onto cork and fixed in Bouin's solution for 6 - 12 hours (longer fixation makes the specimen brittle and difficult to cut on the microtome).

They were then processed and embedded in paraffin wax. Serial sections were cut at 5 μm thickness and mounted on glass slides. Two series of slides were prepared from each block; these were stained with P.A.S. and H.E.

RESULTS OF MORPHOLOGICAL STUDY

THE NON PREGNANT UTERUS

The rabbit uterus is bicornuate with separate cervices opening into a common vagina. In a uterus which has been opened along the antimesometrial border (Fig. 1) one can see pairs of endometrial folds. The largest of these lie on the mesometrial border (Fig. 2) and are named the placental folds; adjacent to these are the periplacental folds and in the antimesometrial wall there are the obplacental folds. Each set of folds is divided into rectangular blocks of endometrium known as the coussinets of Hollard, which are each 2 - 3 mm in length.

The vascular endometrial stroma is covered by the uterine epithelium which is made up of simple columnar cells (Fig. 3). Simple tubular mucus secreting glands are prevalent at the bases of the various fissures. The endometrium is surrounded by a layer of smooth muscle - the myometrium.

DETAILS

In Fig. 4 the complex fold pattern of the endometrium is shown. It consists of both radial and longitudinal folds. The uterus has been opened along the mesometrial border and a small piece excised from the middle of the uterus. The oviductal end lies on the left. Two coussinets of a placental fold are shown separated from the periplacental folds, which are not so clearly demarcated by a deep periplacental fissure. Radial folds join the two sets of longitudinal folds.

The placental folds are fissured. Some of these fissures are undoubtedly artifacts caused by drying. Many round openings are present on the surface of the radial folds and an example of one of

these is shown in Fig. 5. These openings, which have a diameter of 25 μm, are the mouths of simple tubular glands which are prevalent in the periplacental fissure. Individual epithelial cells are arranged radially or possibly helically in the wall of the tube. There are a large number of ciliated cells in the vicinity of the gland opening.

THE SEVEN DAY PREGNANT UTERUS AND BLASTOCYST

Fig. 6 shows the earliest stage of implantation of the blastocyst. The uterus has a diameter of 1.1 cm. The cut edge of the mesometrium lies below in the photograph. The placental folds are enlarged, flattened and separated by a deep irregular interplacental fissure. The periplacental folds are also enlarged, but the obplacental folds have been obliterated by the expanding blastocyst. The antimesometrial wall has thinned and the smooth muscle layer has been stretched.

A low power SEM micrograph (Fig. 7) shows that, at the site of implantation, the two placental folds now form a cup shaped area, with an elaborately fissured surface. In section (Fig. 8), this fissuring is seen to be due to folding of the uterine epithelium, with intervening deep furrows. Later, trophoblast grows into these furrows, over a horseshoe shaped site of attachment. The endometrial tunica propria is highly vascular.

The blastocyst is still invested by zona pellucida. It has shrunk away from the antimesometrial wall, but is still attached to the placental folds. The embryonic plate consists of two layers of cells, ectoderm and endoderm, whilst the abembryonic part of the blastocyst consists only of one layer, of ectoderm, at

this stage. Endoderm rapidly grows round the abembryonic hemisphere, thereby forming the bilaminar omphalopleure.

Scattered clumps of cilia are seen on the epithelium of the cup shaped placental folds (Fig. 9). (Some of the fissures seen in this photograph are artifacts caused by drying). The uterine surface of individual cells is shown in Fig. 10. The cilia are approximately 15 μm long. The other cells bear microvilli, which have small aggregates of coagulum attached to them.

THE NINE DAY CONCEPTUS

Fig. 11 shows a vertical section through the conceptus at its site of attachment to the placental folds. The embryo itself is situated centrally, overlying the interplacental fissure, and with its dorsal surface facing down in the photograph. The neural tube, pharynx and heart are seen. Attached to its lateral margins is the trilaminar omphalopleure, a membrane consisting of an outer layer of trophoblast, an inner layer of endoderm and an intervening layer of vascular mesoderm. The trophoblastic ectoderm and the vascular mesoderm together constitute the chorion, which is attached to the placental folds. The endometrium is highly vascular and is rapidly thickening. In some areas, the trophoblast is attached to, and beginning to invade, the endometrium of the placental folds (Fig. 12).

The relationship between trilaminar omphalopleure and maternal tissues over an area of non-attachment is shown in Fig. 13, uppermost in the photograph are the endodermal cells, whose vacuolated apices face upwards into the cavity of the yolk sac. Their vacuolation suggests that at nine days they may be absorbing

material from the yolk sac cavity. Lying below the endoderm is the mesoderm, in which there are vitelline blood vessels, containing nucleated foetal blood cells. The foetal circulation is established at about this time. The mesoderm seems to form two well defined layers. The upper layer will form, with the endoderm, the definitive visceral yolk sac; the lower layer is associated with the trophoblast which at nine days is made up of a regular layer of cuboidal cells.

In the context of the present study it should be noted particularly that the endoderm, mesoderm and mesodermal derivatives are separated from the maternal tissues by a layer of trophoblast at the nine day stage. "Separation" may be taken to imply "immunological isolation" at this stage.

THE TEN DAY CONCEPTUS

Fig. 14 shows a section through the embryo at the level of the heart, pericardial cavity, pharynx and hind brain. [Note that the embryo has ruptured the investing yolk sac during processing and should be in an inverted position.] The amnion, which developed on the ninth day, completely invests the embryo.

A large vessel, the sinus terminalis, is evident at the edge of the trilaminar omphalopleure and marks its boundary with the bilaminar omphalopleure. (Figs. 15 and 16). This consists of trophoblast and flattened endoderm.

At all points in both the trilaminar and bilaminar omphalopleures, trophoblast separates the other components of these membranes from maternal tissues.

The relationships between the trilaminar omphalopleure,

bilaminar omphalopleure and maternal tissues are shown in Fig. 15. The uterine epithelium is symplasmic except for the deeper parts of the uterine glands.

The visceral yolk sac is peeling off the chorionic layer overlying the placental folds, thereby forming a cavity - the exocoelom. It is interesting to note that the major vessels have formed where the trophoblast of the trilaminar omphalopleure has not invaded the maternal tissues (Fig. 16).

THE THIRTEEN DAY CONCEPTUS

Figs. 17 and 18 are cross sections of the uterus and conceptus at the thirteenth day of pregnancy. Fig. 17 is a thick section, photographed under incident light. The embryo is now well developed. The tail and hind limb buds are wrapped around the allantoic diverticulum. The vitelline diverticulum runs upwards to the visceral yolk sac. Both of these diverticula consist of endoderm, mesenchyme and blood vessels which carry blood to and from the foetus. Thus two extra-embryonic circulations are established, one, the vitelline circulation which serves the visceral yolk and carries nutrients absorbed from the uterine cavity, the other, the chorioallantoic circulation which serves the chorioallantoic placenta and carries nutrients absorbed directly from the maternal blood.

The endometrium has continued to thicken. The placental folds form highly vascular cushions, separated by a deep interplacental fissure. The extent of the trophoblast invasion is clearly shown in Fig. 18. Deep within the interplacental fissure a chorionic membrane joins and demonstrates the depth of trophoblast invasion

into the placental cushions. The periplacental folds show a marked decidual reaction and are highly vascular. The uterine cavity contains a mixture of uterine milk and a darkly staining slough of symplasma and bilaminar omphalopleure.

The arrangement of the foetal membranes is best seen in Figs. 18 and 19. Details of Fig. 18 are shown in Figs. 20 and 21. The embryo lies surrounded by the amnion and anchored in position by the allantoic and yolk sac diverticula. The amnion forms on the ninth day by the development and fusion of ectodermal folds. It consists of an inner layer of flattened ectoderm and an outer layer of mesenchyme (Fig. 20), and separates the embryo from the visceral yolk sac. The large cavity between the amnion and visceral yolk sac is the exocoelom which formed during inversion of the visceral yolk sac during the tenth and eleventh days.

The visceral yolk sac contains numerous vessels which radiate to and from the central yolk stalk (Fig. 19). The gestation sac has been opened by a cruciate incision in the antimesometrial wall of the uterus, which has retracted to reveal the embryo covered by the visceral yolk sac. This is an early stage in the dissection and removal of the yolk sac for the grafting experiments. The sinus terminalis is seen in Fig. 21 coursing around the periphery of the visceral yolk sac. In order to remove only yolk sac it is necessary to remain within the bounds of this vessel, but it should be noted that in parts, the sinus terminalis is not continuous and inadvertent removal of the paraplacental chorion along with the visceral yolk sac may occur.

The relationships between the visceral yolk sac, the paraplacental

chorion, the remaining bilaminar omphalopleure and the maternal tissues is shown in Figs. 18 and 21. The visceral yolk is completely inverted by day 13. Its peripheral border is attached to the paraplacental chorion which in turn is attached to the periplacental fissure. The paraplacental chorion consists of an outer layer of cuboidal trophoblastic cells which are continuous peripherally with the trophoblast of the remaining bilaminar omphalopleure and centrally with the trophoblast invading the placental cushions. Deep to the trophoblast there is a layer of flat mesenchymal cells. This layer is later reinforced by an outgrowth of mesoderm originating from the allantoic diverticulum, it carries with it blood vessels which form anastomoses with the vessels of the visceral yolk sac. Thus the vitelline and chorio-allantoic circulations are joined.

The peripheral margin of the visceral yolk sac is separated from the maternal tissues by the remaining bilaminar omphalopleure. This consists of an outer layer of flat trophoblastic cells and an inner layer of flat endodermal cells. The cavity of the yolk sac intervenes between the visceral layer of the yolk sac (trilaminar omphalopleure) and the parietal layer of the yolk sac (bilaminar omphalopleure).

In summary, the peripheral part of the visceral yolk does not make direct contact with the maternal tissues. It is prevented from doing so by two named membranes, both of which are orientated such that trophoblast is the only tissue which can make direct contact with the maternal tissues.

Relationships between the antemesometrial wall and the conceptus at seven days

During the sixth and seventh days the blastocyst expands rapidly, resulting in stretching of the antimesometrial wall of the uterus and obliteration of the obplacental folds. In Fig. 22 which is a section of part of the antimesometrial wall at the seventh day, the epithelium consists of a single layer of columnar cells lying on a thin layer of endometrial stroma. Simple mucus secreting glands are present, their deepest parts lying in contact with the myometrium.

At seven days the blastocyst is surrounded by the zona pellucida which is in direct contact with the uterine epithelium. This contact is not seen in Fig. 22, the blastocyst wall having been displaced in processing. However, the imprint of the expanded blastocyst is shown by the flattened appearance of the superficial folds of endometrium in Figs. 22 and 23. Fig. 23 is a low power SE micrograph and shows the complex and irregular fold pattern of the antimesometrial wall. Only occasional cilia are present as compared with the uterine epithelium of the placental folds. (Fig. 9).

In summary, the extraembryonic region of the blastocyst is associated with the antimesometrial wall. It consists of the bilaminar omphalopleure, an inner layer of endoderm, an outer layer of trophoblast, and a single extracellular coat - the zona pellucida, which separates the cellular layers of the blastocyst from the maternal uterine epithelium. The pressure exerted by the expanding blastocyst on the antimesometrial wall is indicated by the flattened appearance of the superficial folds of endometrium.

Nine days

The uterine epithelium has formed a symplasma except for the deeper parts of the simple tubular glands which have retained their cellular structure (Fig. 24). The zona pellucida has disappeared and trophoblast knob formation with penetration of the endometrial stroma has occurred. Obplacental giant cells are seen lying just below the symplasma. They appear to be related to the sites of trophoblast attachment to symplasma.

The extra embryonic blastocyst wall consists of a bilaminar omphalopleure made up of an outer flattened layer of trophoblastic epithelium which is attached to the uterine symplasma at discrete points. Between these sites of attachment the bilaminar omphalopleure forms arcades. The inner layer consists of a flattened layer of endodermal cells.

In summary, the nine day antimesometrial region is characterised by the appearance of obplacental giant cells at the sites of trophoblast attachment. The endoderm of the bilaminar omphalopleure is separated from the maternal uterine symplasma by a continuous layer of trophoblast.

Ten days (Fig. 25)

The stroma of the endometrium is very vascular. The uterine symplasma appears to be degenerating and several of the arcades of bilaminar omphalopleure have broken and sloughed into the uterine lumen. The obplacental giant cells now lie deep in the endometrial stroma and are closely related to the myometrium.

In summary, the bilaminar omphalopleure and associated uterine symplasma slough into the uterine lumen during the tenth day, thus

converting the yolk sac cavity and uterine lumen into a single cavity. The endoderm of the bilaminar omphalopleure does not at any stage make direct contact with the antimesometrial endometrium.

Thirteen days (Figs. 26, 27, 28)

The uterine lumen contains uterine milk consisting of glandular secretions and the remains of the sloughed symplasma and bilaminar omphalopleure. The endometrial stroma is still highly vascular. The uterine symplasma has been replaced by growth and migration of cells from the deep parts of the simple uterine glands. The uterine epithelium now consists of a single layer of columnar epithelial cells bearing long regular microvilli. No ciliated cells were seen (Fig. 28).

The fold pattern of the endometrium is regular and seems to be different from that at seven days (Fig. 27). However, this may be due to stretching of the specimens during fixation. In Fig. 27 many of the epithelial cells are covered by an amorphous layer of uterine milk and cell debris.

In summary the thirteenth day is characterised by the appearance of large obplacental giant cells, some of which seem to have migrated into the myometrium. The endometrial folds are not flattened as they were at the seven day stage. So it seems that the visceral yolk does not exert pressure on these folds, and indeed in the histological preparations the visceral yolk sac was always separated from the uterine epithelium by a space - the uterine lumen. This may be explained by the fact that the visceral yolk sac shrinks away from the uterine epithelium during tissue

fixation.

What is transplanted?

Having outlined the development of the yolk sac and its relationship to the endometrium, we now describe the definite structure of the material transplanted as "visceral yolk sac". It consists of mesodermal and endodermal derivatives in three layers and Fig. 20 demonstrates these: an outer layer of columnar endodermal cells with vacuolated apical cytoplasm and basally situated nuclei, an intermediate layer of vitelline vessels and nucleated foetal blood cells and an inner layer of flat mesenchymal cells. The outer layer faces the uterine lumen and the inner layer faces the exocoelom.

The surface of the endoderm consists of regular sized cells (Fig. 29). The apex of each cell is dome shaped and bears irregular short microvilli (Fig. 30). The surface of the inner mesenchymal layer is flat and is characterised by ridges created by underlying vitelline vessels (Fig. 31).

Greater detail is obtained from the transmission electron micrographs of 13 day visceral yolk sac, shown in Figs. 32, 33, 34, 35 and 36. Each columnar endodermal cell rests on a thin basal lamina and is surrounded by an intact cell membrane. Adjacent cells are united at the luminal surface by junctional complexes, the deeper parts of the membranes are joined by desmosomes. The dome shaped apices are covered by branching microvilli of length 0.7 - 0.8 μm. Directly beneath this microvillous border there is a complex of tubules, pinocytotic vesicles and lysosomes. This is similar to the canalicular system in rat yolk sac described

by Padykula (1966).

The apical cytoplasm contains numerous absorption droplets which vary in electron density. It is interesting to note that the cytoplasm of adjacent cells varies in electron density possibly reflecting some functional difference between cells or possibly some form of cyclical change in an individual cell. Occasional mitochondria are present in the apical cytoplasm.

The nucleus contains two or three nucleoli, and occasionally a few absorption droplets. The endodermal cells show mitotic activity at 13 days.

The cytoplasm between the nucleus and basal lamina contains numerous mitochondria and almost all of the rough endoplasmic reticulum of the cell. The basal plasmalemma is flat. Occasionally vacuoles and inclusion bodies associated with mitochondria are seen lying closely related to the basal plasmalemma.

Deep to the basal lamina of the endodermal layer this is a space filled by a loose arrangement of collagen fibres. The vitelline vessels are formed by a continuous layer of endothelial cells and contain nucleated foetal blood cells. The spaces between vitelline vessels are filled by collagen fibres, ground substance and phagocytic cells which Slade (1969) calls macrophages.

The inner layer is composed of a mesothelial layer resting on a basal lamina. The luminal surface contains a few microvilli.

In summary the visceral yolk sac is a complex membrane composed of three layers. The endodermal cells are highly specialised. The morphological details reflect their function which is absorption

of materials from the uterine lumen. Several facts suggest that the visceral yolk sac does not make direct contact with the uterine epithelium.

(i) The endometrial folds of the antimesometrial wall are not flattened as they should be if the visceral yolk sac was was held against the uterine epithelium by pressure.

(ii) Both the uterine epithelial cells and yolk sac endodermal cells have dome shaped apices. Again this suggests that the visceral yolk sac does not exert pressure on the uterine epithelium.

(iii) The microvilli of the uterine epithelium and endodermal cells are upstanding. Interdigitation between the two sets of microvilli seems unlikely because of their differing structure. The uterine epithelium consists of long regular microvilli whilst the yolk sac endoderm has short branched microvilli.

(iv) If there were intercellular junctions between uterine epithelium and endodermal cells one would expect the microvilli to be fragmented.

(v) In all the photomicrographs of other workers and in my own histological preparations the visceral yolk sac is separated from the uterine epithelium by the uterine lumen.

DIAGRAM 2

The relationships between the 9 day embryo and surrounding membranes and the maternal tissues

KEY

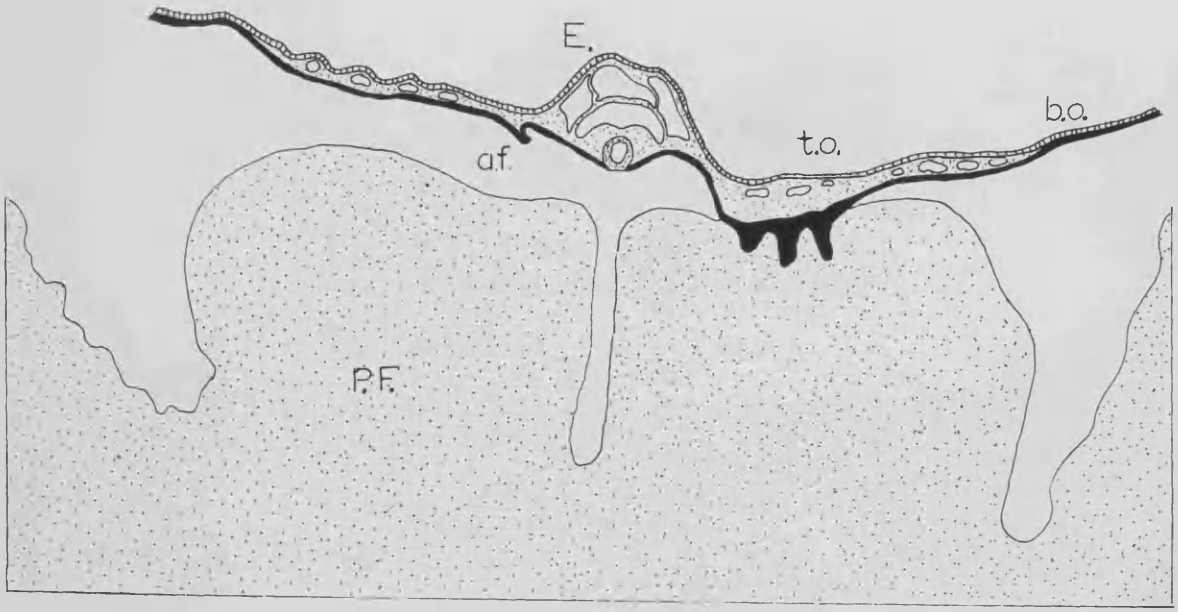
E	=	Embryo
PF	=	Placental fold
to	=	Tri-laminar omphalopleure (developing yolk sac and chorion)
bo	=	Bilaminar omphalopleure (endoderm and trophoblast)
af	=	Amniotic fold
▬	=	Trophoblast

DIAGRAM 3

The relationships between the developing foetal membranes, foetus and maternal tissues at 10 days

KEY

E	=	Embryo
a	=	Amnion (ectoderm and mesoderm)
vy	=	Visceral yolk sac (mesoderm and endoderm)
to	=	Trilaminar omphalopleure
bo	=	Bilaminar omphalopleure
PF	=	Placental fold
▬	=	Trophoblast


E.
a.f.
t.o.
b.o.
P.F.

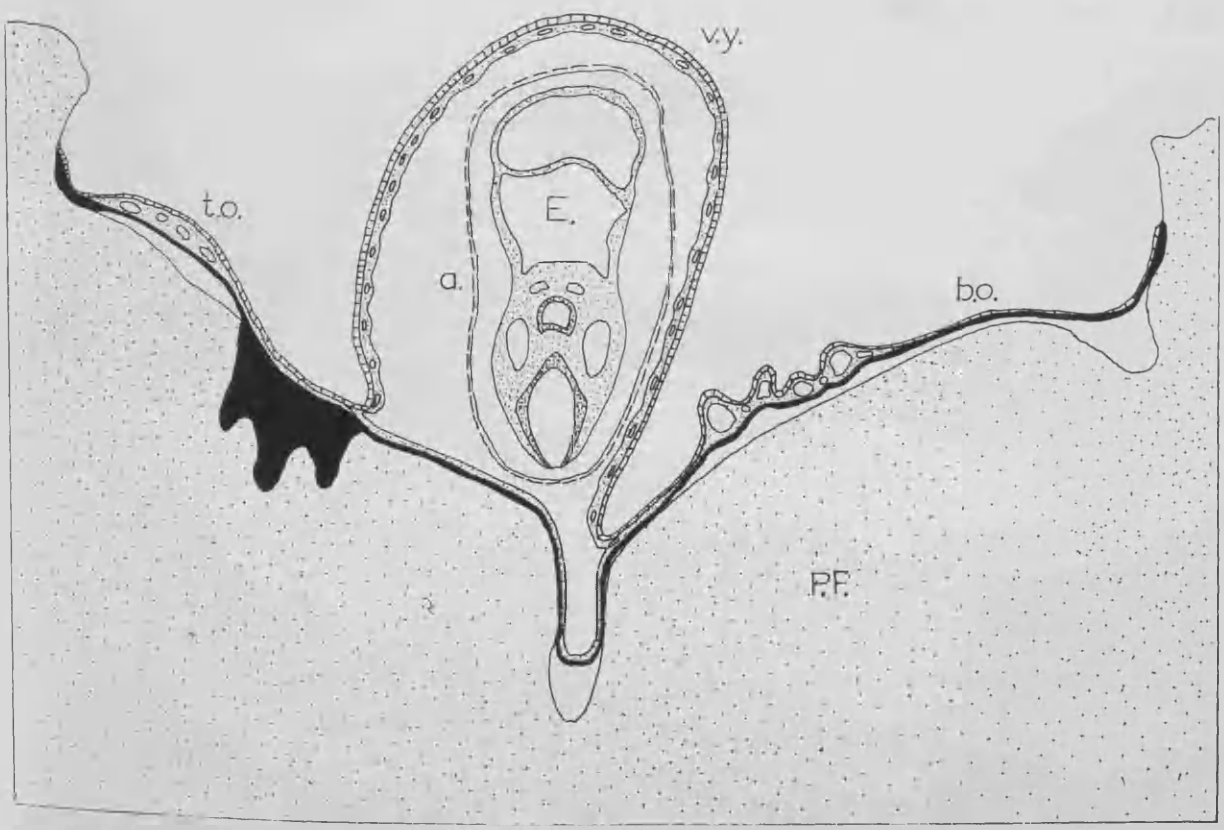
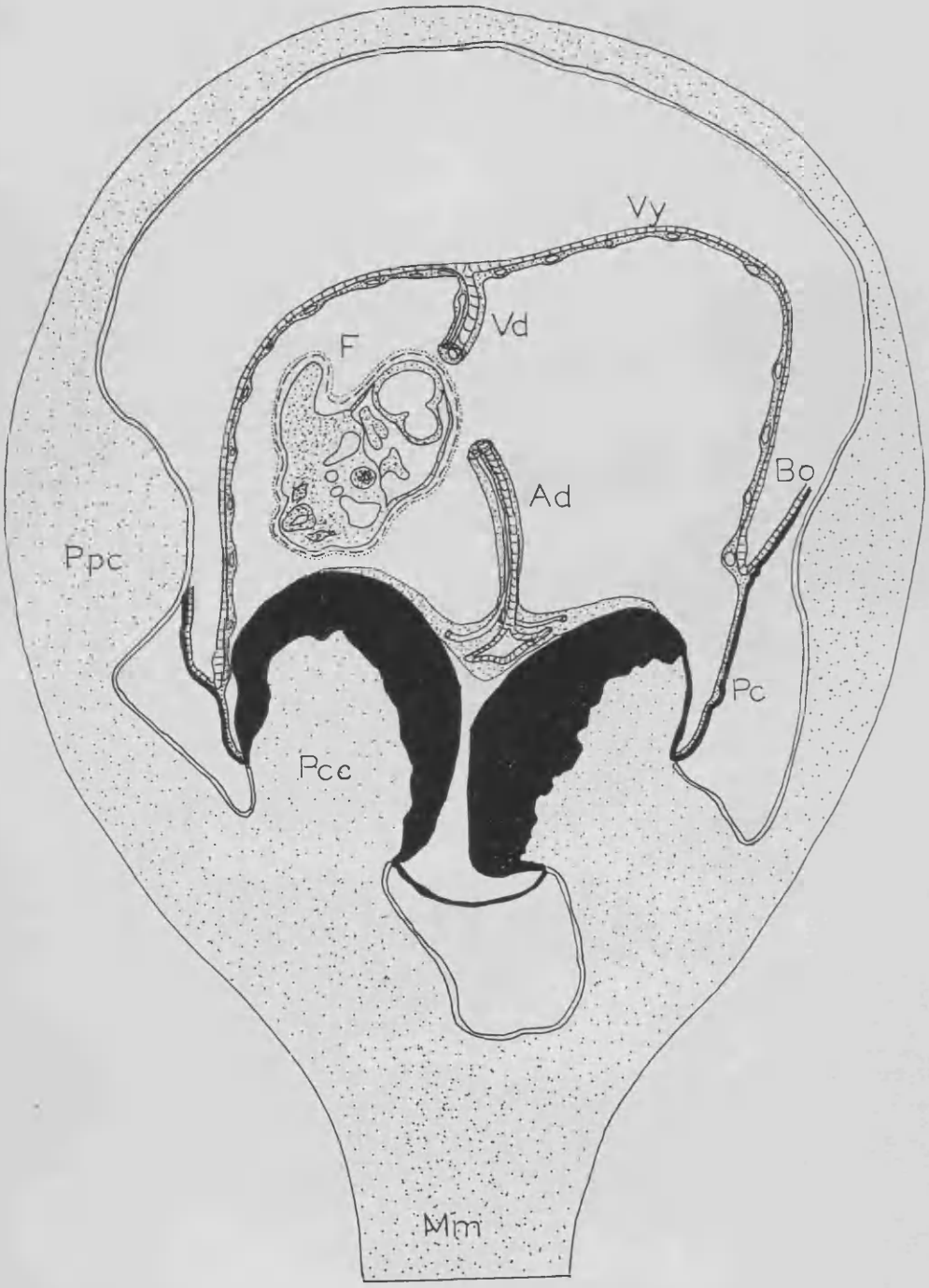

v.y.
t.o.
E.
a.
b.o.
P.F.

DIAGRAM 4

The relationships between the foetal membranes and the maternal tissues at 13 days

KEY

F	=	Foetus
Vv	=	Vitelline diverticulum
Ad	=	Allantoic diverticulum
Vy	=	Visceral yolk sac
Bo	=	Bilaminar omphalopleure remnant
Pc	=	Paraplacental chorion
Pcc	=	Placental cushion
Ppc	=	Periplacental cushion
Mm	=	Mesometrium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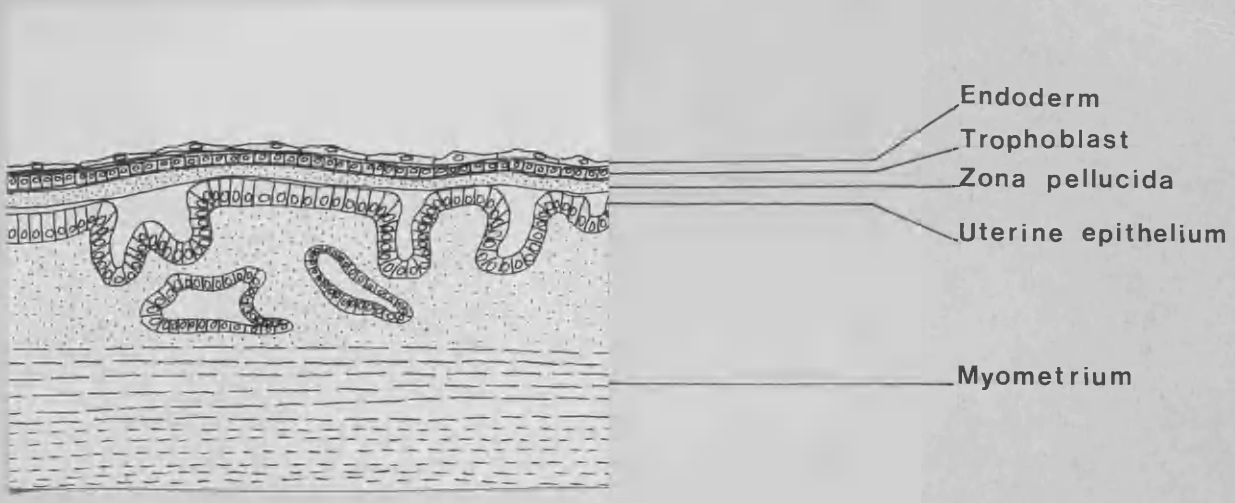
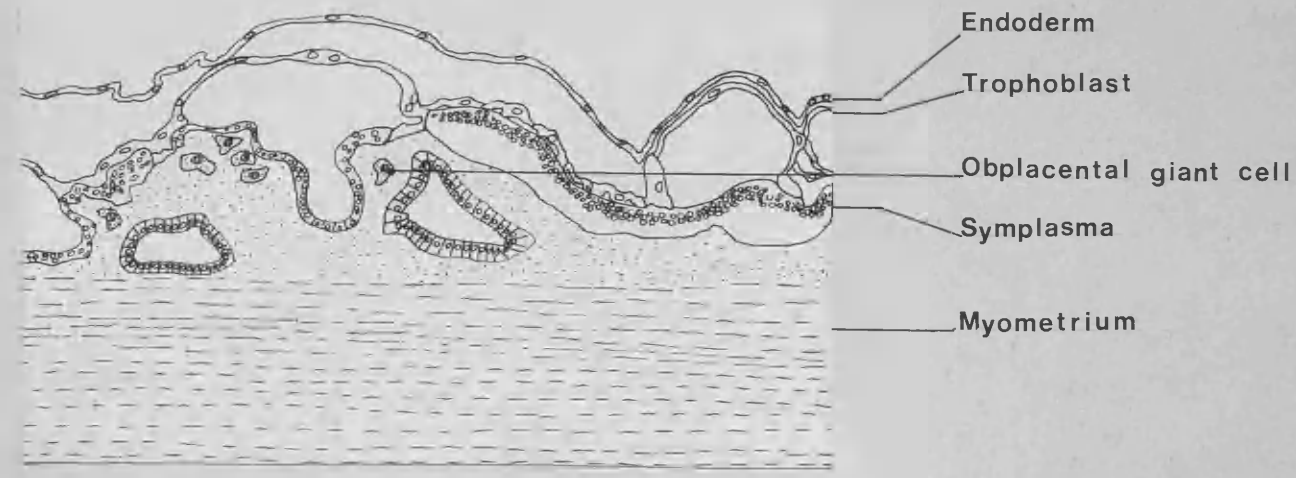
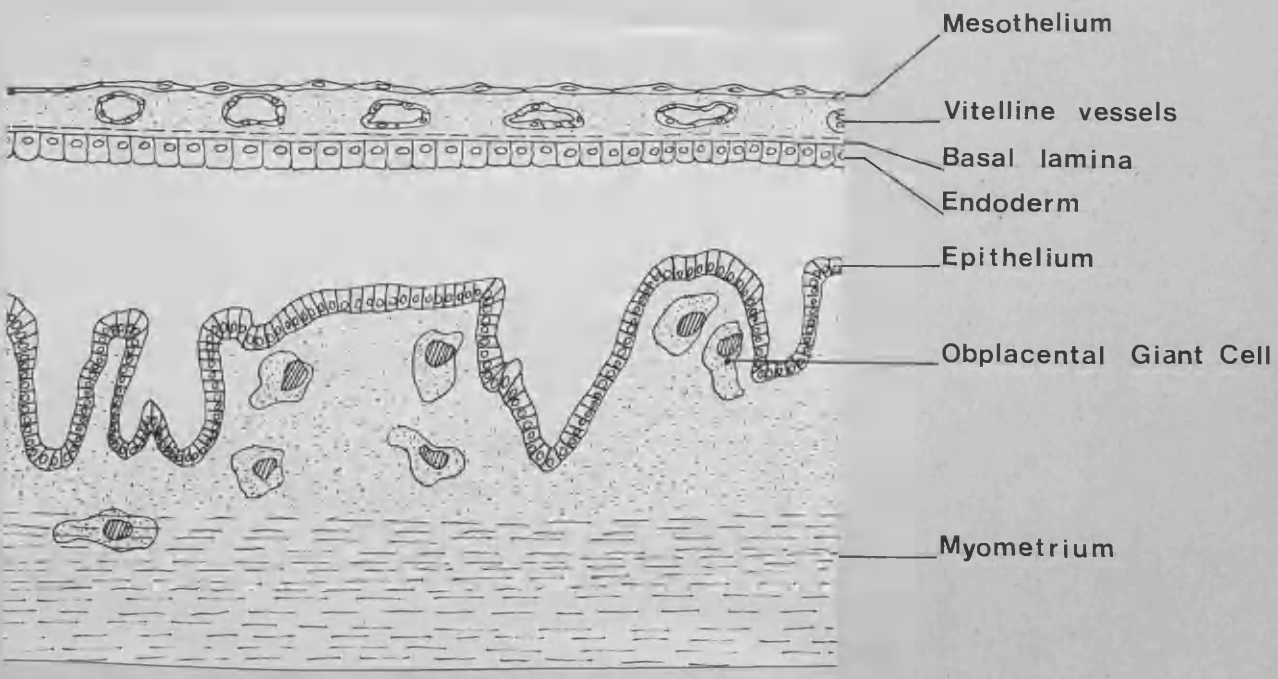
Vd
F
Ad
Bo
Ppc
Pc
Pcc
Mm


Endoderm
Trophoblast
Zona pellucida
Uterine epithelium
Myometrium


Endoderm
Trophoblast
Obplacental giant cell
Symplasma
Myometrium


Mesothelium
Vitelline vessels
Basal lamina
Endoderm
Epithelium
Obplacental Giant Cell
Myometrium

RESULTS OF GRAFTING EXPERIMENTS

GRAFTING EXPERIMENTS

In designing an experiment to assess the presence or absence of transplantation antigens in a particular tissue it is necessary to satisfy three criteria -

(i) The transplanted tissues must not be placed in an immunologically privileged site.

(ii) The graft dose must be sufficiently high to stimulate an allograft rejection response.

(iii) The transplanted tissues must be viable at the time of grafting and must not succumb to non-specific factors, such as poor vascularisation or infection.

Each of these criteria was examined with the following results.

The graft site - The structure of the rabbit ear

A plate of elastic cartilage forms the skeleton and provides support for the ear. Immediately dorsal to the perichondrium which surrounds the cartilage there is a subcutaneous layer composed of loose areolar tissue containing large neurovascular bundles. These branch into the overlying dermis of the dorsal skin layer and through perforations in the elastic cartilage into the dermis of the ventral skin layer.

As described in Materials and Methods, it was aimed to place the grafts in the dermis of the dorsal skin layer, and most of them were in fact recovered from this layer. Medawar (1944) and many others have shown that this dermal layer is not an immunologically privileged site; should graft survival be extended therefore, it must be due to some other factor.

The graft dose

The approximate number of cells grafted was estimated using the SEM pictures of yolk sac. Fig. 20 demonstrates that endoderm lies as a single layer of cells which are individually demarcated by clear intercellular grooves. Using Fig. 29 and other lower power photo-micrographs it was calculated that there are approximately 24,000 endodermal cells/sq. mm of visceral yolk sac. (This estimate does not take into consideration the perspective effect of the scanning electron micrographs).

The total area of visceral yolk sac at 13 days was estimated from Fig. 18 and is about 125 sq. mm. Each graft consisted of $\frac{1}{2}$ - $\frac{3}{4}$ of a single yolk sac, thus about $1.5 - 2.0 \times 10^6$ endodermal cells were grafted into each ear of a single rabbit. The total number of cells grafted into each rabbit must also include cells of the other two layers which make up the yolk sac membrane. Presuming an approximate ratio of one endodermal cell to 2 or 3 cells of mesodermal origin each rabbit received $6 - 9 \times 10^6$ cells.

Viability of grafts

Samples of excised yolk sacs were fixed in Bouin's solution after about $\frac{3}{4}$ hour in T199 Medium. Fig. 37 shows that the endodermal cells are morphologically intact and have retained the same appearance as those cells which were fixed immediately after excision (Fig. 20). Grafting was always carried out within $\frac{3}{4}$ hour of excision from the donor rabbit. It is noteworthy that Fig. 37 shows that a small amount of paraplacental chorion and possibly bilaminar omphalopleure is grafted with the visceral yolk sacs. It was not possible to determine which grafts included

these other membranes. It should also be noted that in Fig. 37 most of the vitelline vessels are empty of foetal blood cells.

Grafts were removed, together with surrounding host tissues, at 6, 9 or 14 days after grafting. The numbers removed at each stage are summarised in the following Table

TABLE TO SHOW INTERVAL BETWEEN GRAFTING AND REMOVAL

GRAFT NO.	INTERVAL	TOTAL
D1 - D2	6 days	10
F4 - F6	6 days	
G1 - G3	9 days	6
C1 - C2		
E1 - E3	14 days	16
F1 - F3		
		32

Each graft was given a code number e.g. D.1.R., according to the following scheme.

CODING OF GRAFT NUMBERS

1. First letter refers to experiment number. All experiments under the same letter were performed on the same day.
2. Second number refers to the rabbit used in a particular experiment.
3. Third letter refers to the ear (right or left) from which the graft was removed.

The status of each graft was assessed histologically, on a semi quantitative basis, using the following conventions.

0 = no surviving cells found

\+ = ∧

++ = ∧

+++ = ∧

Pleomorphic cells are cells presumed to be of endodermal origin. Two variants were seen, flat types which lay in continuity with normal living endoderm lining graft cysts, and, rounded types which lay isolated on the wall of a cyst.

ASSESSMENT OF STATUS OF GRAFTS

Six days (10 Grafts)

Graft No.	Living Endoderm	Pleomorphic Cells	Dead Endoderm
D1R	+++	+	++
D1L	++	++	+++
D2R	+++	0	++
D2L	++	0	+++
F4R	+++	++	++
F4L	+++	0	+
F5R	++	+	++
F5L	++	0	++
F6R	+++	+	+
F6L	++	+	+
TOTAL	(100%)	(60%)	(100%)

Nine days (6 Grafts)

Graft No.	Living Endoderm	Pleomorphic Cells	Dead Endoderm
G1R	++	++	+++
G1L	++	+	+++
G2R	0	++	+++
G2L	0	++	++
G3R	+	+++	++
G3L	0	+++	++
TOTAL	(50%)	(100%)	(100%)

Fourteen days (16 Grafts)

Graft No.	Living Endoderm	Pleomorphic Cells	Dead Endoderm
C1R	0	+	+++
C1L	0	0	++
C2R	0	0	++
C2L	0	0	++
E1R	0	0	++
E1L	0	0	+++
E2R	0	0	++
E2L	0	++	++
E3R	0	+	+++
E3L	0	0	+++
F1R	0	+	++
F1L	0	+	++
F2R	+	+	++
F2L	0	0	++++
F3R	0	0	++
F3L	0	0	++
TOTAL	(6%)	(37%)	(100%)

HISTOLOGICAL APPEARANCES

The following section summarises the histological appearances of each graft.

A. GRAFT REMOVED IMMEDIATELY AFTER IMPLANTATION (Fig. 38)

This served as a control of the histological appearances of both the normal yolk sac at the time of grafting, and of the graft site.

The graft lies in the deepest part of the dermis of the rabbit ear. Epidermis is seen above the graft and deep to the graft there is a medium sized artery lying in the subcutaneous tissues.

The endodermal cells of the graft have retained their columnar appearance. The apical cytoplasm of each cell is vacuolated. Foetal blood cells with dark stained nuclei are distributed throughout the graft. The grafted cells are packed closely together.

B. GRAFTS REMOVED AT 6 DAYS AFTER GRAFTING

DIR

The graft lies in the deep part of the dermis, overlying a medium sized artery and the elastic cartilage plate of the rabbit ear. (Fig. 39). It shows healthy endodermal cells and a central mass of tissue which appears to consist of necrotic endodermal cysts filled with eosinophilic debris. Giant cells are present around the deep parts of the graft. Surviving endodermal cells are cuboidal and do not have vacuolated apical cytoplasm. (Fig. 40).

The main mass of giant cells is shown in Fig. 41, adjacent to a small artery which is cuffed by a round cell infiltrate.

Note that at least one of the giant cells is multinucleated and each cell is surrounded by a PAS positive border.

D1L

A single graft cyst is present, lined by columnar endodermal cells and flattened cells which are pleomorphic variants of endodermal cells. Most of the graft appears to be dead. A massive eosinophilic mass infiltrated by small lymphocytes is present to the left and below the cyst.

D2R

The centre of the graft is composed of an eosinophilic mass containing pycnotic nuclei and a heavy infiltrate of small lymphocytes. Surrounding this mass there are two large graft cysts lined by apparently healthy endodermal cells. Giant cells are present and lie some distance from the main mass of graft, suggesting that they have migrated away from the graft.

D2L

The graft consists of a central mass of eosinophilic cell debris, pycnotic nuclei and infiltrating lymphocytes. In places, the lymphocytes are packed together and appear to be intravascular.

Living endodermal cells are seen forming cysts around the superficial part of the graft. A mass of giant cells lies to one side of the graft. They appear to have migrated away from the graft.

There is a moderately heavy infiltrate of small lymphocytes, macrophages, eosinophils and occasional polymorphs.

F4R

Several large cysts are present in this graft infiltrated by lymphocytes and in various stages of destruction. Fig. 43 shows one of the cysts, adjacent to the main mass of graft. It is lined on its superficial surface by columnar cells whilst on its deep surface there are only occasional pleomorphic variants. Figure 44 shows a superficial cyst lined on its upper (superficial) surface by cuboidal endodermal cells. On its deep surface the lining is broken by eosinophilic cell debris, containing pycnotic nuclei and invading small lymphocytes.

F4L

In Fig. 45 several graft cysts are shown lined by surviving endodermal cells, which are either columnar or cuboidal cells, or pleomorphic variants. A heavy lymphocytic infiltrate is present in the centre of the graft. Fig. 46 is a detail of Fig. 45 and shows the various cell types. The spindle cells are presumably either mesodermal cells or fibroblasts. Fig. 47 shows endodermal cells which have rounded and separated from one another, no longer forming a continuous epithelial sheet. As judged by their nuclear morphology they are still surviving. Some lie as free cells within the cyst cavity.

F5R

Living endodermal cells are seen forming a cyst. (Fig. 48). A heavy infiltrate of lymphocytes and macrophages surrounds the graft.

F5L

A graft cyst has formed lined by a variety of cells, which

are judged histologically to be surviving. There is a moderately heavy infiltrate of lymphocytes, eosinophils and macrophages around the graft.

F6R

A graft cyst is shown lined on its deep surface by pleomorphic variants of endodermal cells and on its superficial surface by an irregular line of cubical and low columnar endodermal cells which are eosinophilic (Fig. 49). Note especially the vessel in the main necrotic part of the graft which is packed with small lymphocytes. This vessel may be a recannalised vitelline vessel or an ingrowing vessel of host origin.

Fig. 50 shows a detail of part of a cyst, and show surviving endodermal cells.

F6L

Fig. 51 shows the main central mass of the graft to be necrotic. But lying around this there are cysts containing living endodermal cells. The distribution of infiltrating cells is typical: from above cells infiltrate from the capillary plexus underlying the epidermis and from below cells infiltrate from the perichondrial vessels.

C. GRAFTS REMOVED AT 9 DAYS AFTER GRAFTING

G1R While most of the graft is necrotic and heavily infiltrated by lymphocytes and occasional macrophages, a cyst lined by surviving endodermal epithelial cells is present.

G1L

Living endoderm is present, but again most of it appears necrotic. There is a heavy infiltrate of lymphocytes and a moderate

number of eosinophils. Giant cells are present.

G2R

Endoderm is seen, but many of the cells are necrotic, flattened against the wall of the cyst or shed into the cyst lumen. There is a heavy infiltrate of lymphocytes and many of the macrophages appear to have taken up PAS positive material, presumably the remains of dead graft tissue.

Sections through the puncture site where the cannula has traversed the skin show a tube of epidermis growing down towards and investing, necrotic graft.

G2L

The surrounding infiltrate is heavy and consists of small lymphocytes and macrophages. The latter again contained PAS positive granules. Only flattened cells are seen lining the cyst. Occasional lymphocytes infiltrate the necrotic graft tissue.

G3R

Flattened and columnar endodermal cells line the cysts. Lymphocytes have infiltrated the graft and lie amongst the necrotic debris. There are many pycnotic nuclei and what appear to be masses of dead foetal blood cells.

G3L

Flattened cells line the graft cyst. A heavy lymphocyte infiltrate is present.

D. GRAFTS REMOVED AT 14 DAYS AFTER GRAFTING

C1R

The graft is completely destroyed. (Fig. 52). The infiltrate

consists mainly of small lymphocytes and macrophages.

Fig. 53 shows the remains of part of the yolk sac. The basal lamina is PAS positive and lying on its convoluted surface are the ghosts of necrotic endodermal cells. The cyst cavity contains necrotic debris and occasional small lymphocytes. The graft to the left is necrotic and occasional macrophages are seen.

C1L

No living graft was found. A light infiltrate of lymphocytes is shown in the picture (only a small amount of graft appeared to have been transplanted in this case).

C2R

No living graft tissue is seen. (Fig. 55). Most of the graft is strongly eosinophilic, and does not contain lymphocytes, but there is a heavy infiltrate of small lymphocytes around the grapt.

C2L

No living endodermal cells are seen. (Fig. 56). The graft is heavily infiltrated by small lymphocytes. Epidermis is seen on the left of the picture. This has grown down as a tube from the puncture site and invested the necrotic graft.

E1R

The graft is necrotic. There is a heavy lymphocytic infiltrate. Fig. 57 shows giant cells which lie away from the main necrotic mass.

E1L

No typical endodermal cells are seen. The main necrotic mass

contains the basal lamina of the dead yolk sac. A heavy lymphocytic infiltrate surrounds the dead graft. (Fig. 58).

E2R

Only a small necrotic piece of graft is found surrounded, by a light lymphocyte infiltrate.

E2L

The necrotic graft is surrounded by an epidermal tube in most sections. The graft is eosinophilic and contains many pycnotic nuclei. At the distal end of the epidermal tube the graft lies free in the dermis. It is surrounded by a heavy lymphocyte infiltrate.

E3R

The necrotic graft is enveloped by a tube of epidermis. The invested graft is eosinophilic and contains many pycnotic nuclei. Fig. 60 shows a detail of the remains of the graft, which again is merely an eosinophilic mass at the distal open end of the epidermal tube.

E3L

The graft is totally destroyed. The convoluted basal lamina of the yolk sac is seen on PAS stained sections. A heavy lymphocyte infiltrate surrounds the graft. (Fig. 61).

Giant cells lie in the periphery of the necrotic graft. Figs. 62 and 63 show two such cells surrounded by a heavy lymphocyte infiltrate. There is a thick PAS positive border around each cell.

F1R

The graft is again surrounded by an epidermal tube. Fig. 64

shows the open end of the tube. The graft has been destroyed. A heavy lymphocytic infiltrate is present within the necrotic graft.

F1L

The graft is dead and only the convoluted basal lamina with ghost endodermal cells remains. (Fig. 65). A heavy lymphocytic infiltrate is present in the surrounding tissues.

F2R

Again most of the graft is dead (Fig. 66), but there are some relatively large groups of endodermal cells still recognisable as such. Most of these lie in the middle of the necrotic graft. Their nuclei are mostly pyknotic, but some may belong to surviving cells (Fig. 67).

F2L

No living endodermal cells are seen. There is a heavy cellular infiltrate around the graft site. (Fig. 68).

F3R

No living graft cells are seen. Again the graft is partly enveloped by a tube of epidermis.

F3L

No living endodermal cells are seen. The graft is infiltrated by lymphocytes and giant cells are present in an old graft cyst. (Fig. 69).

DISTRIBUTION AND MORPHOLOGY OF THE GRAFT GIANT CELLS

Giant cells were not found in all the grafts. The Table shows the distribution of these cells in the grafts studied.

The cells usually lie at the periphery of the main mass of the graft and are often at some distance from the graft remains. At 14 days they are never seen in the main mass of the necrotic graft.

The cells appear to be migratory. They are often solitary (Fig. 63), but may occasionally be clumped together, (Fig. 57) and vary in size and shape (Fig. 41). As measured from a single section the diameter of the cells varies between 60 and 80 μm. They may be rounded, or have long cytoplasmic processes. The nucleus lies in the centre of the cell and stains strongly with haemotoxylin. They are occasionally multinucleated. The surrounding cytoplasm stained faintly with haemotoxylin, appears fibrillary in nature. A thick hyaline membrane surrounds each cell. It stains strongly with eosin and PAS reagent and is resistant to diastase digestion. The giant cells are often surrounded by a heavy infiltrate of lymphocytes and occasional macrophages.

TABLE TO SHOW INCIDENCE OF GIANT CELLS IN THE GRAFTS

6 DAYS GRAFTS		9 DAYS GRAFTS		14 DAYS GRAFTS	
D1R	++	G1R	++	C1R	0
D1L	0	G1L	++	C1L	0
D2R	++	G2R	0	C2R	++
D2L	++	G2L	0	C2L	0
		G3R	++		
F4R	0	G3L	++	E1R	++
F4L	0			E1L	++
F5R	0			E2R	0
F5L	0			E2L	0
F6R	0			E3R	0
F6L	++			E3L	++
				F1R	++
				F1L	0
				F2R	0
				F2L	0
				F3R	++
				F3L	0
TOTAL	4 (40%)		4 (67%)		6 (37%)

DISCUSSION

The aim of this investigation was to determine whether transplantation antigens are present in the cells of the visceral yolk sac of the rabbit. The conceptus can be arbitrarily divided into the foetus or embryo and the foetal membranes. Woodruff (1957) had already shown that the rabbit foetus contains transplantation antigens at 16 days. It was therefore of interest to investigate whether the foetal membranes of the rabbit were also antigenic.

The visceral yolk sac was chosen because it does not contain trophoblast and because, after the disappearance of the parietal layer of the yolk sac (bilaminar omphalopleure) it lies in apposition to the maternal tissues without another intervening foetal membrane. Many studies of trophoblast have been undertaken (Billington 1971) and all have shown that membranes which include trophoblast do not normally suffer allograft rejection. Apart from the work of Andrew (1971) and Andrew et al (1975) other foetal membranes have not been studied.

Visceral yolk sac was removed from 13 day rabbit conceptuses because the morphological study had shown that this is the earliest time at which the visceral yolk sac is potentially apposed to the maternal uterine epithelium without the interposition of other foetal membranes.

The site and technique of grafting were satisfactory, in that there was minimal evidence of infection in the grafts and the grafts were easily located, even after 14 days in situ.

Medawar (1944) estimated the median survival time of high dose skin allografts between different strains of rabbits as 7.9 - 12.9 days. The visceral yolk sac grafts were therefore removed at 6,

9 and 14 days after grafting, when theoretically if the visceral yolk sac behaved as an allograft there should be approximately 100%, 50% and 0% of surviving grafts.

The graft dose actually introduced was measured by scanning electron microscopy. However, it is obvious from the appearance of the grafts at 6 days that a large part undergoes non specific ischaemic necrosis. Therefore, the amount of graft actually introduced was not the amount of graft available to stimulate the immune response of the recipient animal. However, the graft dose does appear to have been sufficient to stimulate the immune response as indicated by the presence of a lymphocytic infiltrate and the absence of surviving cells at 14 days. To overcome this problem of ischaemic graft necrosis, it would have been more satisfactory to have made multiple injections of small amounts of graft into each ear. This might have allowed initial survival of more of the graft and provided a larger dose to stimulate the allograft response.

The general appearance of the graft was the same at 6, 9 and 14 days. A central mass of necrotic tissue was surrounded by flattened epithelial cysts which at 6 days were lined with living endoderm and at 14 days were devoid of recognisable surviving endoderm. The central mass was eosinophilic, contained dead cells with many pycnotic nuclei and in some cases the convoluted basal lamina of dead visceral yolk sac, upon which there were the ghosts of endodermal cells. This appearance is attributed to the ischaemic necrosis of the main bulk of the tissue transplanted. On the periphery of this mass the visceral yolk sac

graft received sufficient nutrition from the host to allow its survival.

Despite a large amount of graft necrosis, living endoderm was present in all the grafts removed at 6 days. In most cases the endoderm was located on the side of the cyst nearest to host blood vessels supply. On the side of the cyst adjacent to the central necrotic mass only occasional intact endodermal cells were found. These cells appeared unhealthy and did not form continuous sheets of epithelium. These findings support the idea that the central mass of graft had undergone ischaemic necrosis.

The healthy endodermal cells lining the cyst are cuboidal in shape and form continuous sheets of epithelium. The apices of the cells are not vacuolated as they are in normal, and in situ, yolk sac.

Cuboidal epithelium was seen in every graft, but there were also many pleomorphic variants, which presumably originated from endoderm because they line the cysts and are often found in continuity with a line of cuboidal cells. Two main types of pleomorphic variants were seen: firstly, flattened cells which formed a single layer of epithelium and secondly cells with rounded borders which were usually isolated from the adjacent cells lining the cyst. These rounded cells often appeared unhealthy and some of them were obviously being shed into the lumen of the cyst.

Only half of the excised grafts at 9 days were found to contain living endodermal cells, which again had the same distribution and appearance as in the 6 day grafts.

At 14 days only one graft appeared to contain living endodermal

cells. A sheet of typical endoderm was seen in graft F2R. This piece of visceral yolk sac appears to be healthy. It is situated at the periphery of the central necrotic mass and is surrounded by necrotic material. The graft cysts surrounding the necrotic mass do not contain living endoderm. Although this piece of graft may be lying in an immunologically privileged site, it must be accepted that graft rejection has not occurred in this particular case.

There are three possible causes for this difference in the presence and absence of living endodermal cells at 6, 9 and 14 days.

1. Non specific death due either to ischaemia or infection.
2. Visceral yolk sac endoderm may have an intrinsic life span of less than 14 days when transplanted to the dorsum of a rabbit ear.
3. The endodermal cells have been destroyed by cytotoxic host lymphocytes in an allograft rejection response.

From the previous discussion it is unlikely that the visceral yolk sac once established at the periphery of the necrotic mass should undergo ischaemic necrosis. The giant cells which were found in some of the grafts at 14 days are almost certainly of graft origin. If ischaemia or infection were the cause of endodermal cell death then these cells should also be dead, unless of course, the giant cells are less sensitive to ischaemia or infection than the endodermal cells. There was no evidence that any of the grafts became infected after transplantation.

An unavoidable weakness of this study is the lack of information

on the behaviour and fate of control isografts of yolk sac, made under the same conditions as those used for the allograft series. This would have required pure bred rabbits which are unfortunately not readily available. However, the previous study of Andrew et al (1975) showed extended survival of isografts of mouse yolk sac.

It seems from other work that visceral yolk sac membrane can survive beyond the normal gestational period. Padykula (1964) cultured rat visceral yolk sac in vitro, and found survival of endodermal cells up to 30 days.

The visceral yolk sac of the rat and mouse are similar both morphologically and functionally to the rabbit visceral yolk sac. The visceral yolk sac of the rabbit presumably does not have an intrinsic life span of less than 14 days.

Although adequate control data is not available the visceral yolk sac appears to have undergone an allograft rejection. McLean and Scothorne (1972) made subcutaneous grafts of rabbit skin beneath the skin of the dorsum of the ear. New Zealand white does were used as recipients and coloured Dutch bucks as donors. An isograft series demonstrated healthy grafts after several months. The allografts were found to have encysted, epidermis innermost. Grafts recovered at 8 days were healthy whilst at 9 - 11 days after grafting there was marked lymphocytic infiltration with associated epidermal destruction at 10 - 11 days. Medawar (1944) estimated the median survival time of high dose skin allografts in rabbits as 7.9 - 12.9 days. The timing of visceral yolk sac death is coincident with these findings for epidermal grafts made in rabbits.

A second piece of evidence indicating allograft rejection of the grafts is the heavy lymphocytic infiltrate which was present in all of them. The distribution of lymphocytes was the same in every case. A heavy infiltrate which presumably had originated from the dermal vessels was found lying superficially to the graft. A second infiltrate lay deep to the graft, in close relation to the blood vessels associated with the perichondrium. At 9 and 14 days lymphocytes were also found within the central necrotic mass, occasionally as single cells, but usually as aggregates of cells lying within blood vessels on the periphery of the central necrotic mass. These vessels are either new vessels which have grown into the central necrotic mass or are the remains of vitelline vessels which have recanalised and become anastomosed to the recipient blood vessels.

From this evidence the 13 day visceral yolk sac of the rabbit seems to undergo allograft rejection when transplanted to a subcutaneous site. The discussion so far has been concerned only with the fate of the endodermal cells of the yolk sac. The mesodermal derivatives could never be identified with certainty and therefore have been ignored. The final conclusion regarding the grafting experiments is that the endodermal cells of the visceral yolk sac of the rabbit contain transplantation antigens during the 13th day of gestation. This conclusion is subject to several reservations most of which have already been discussed. One possibility which cannot be excluded on the basis of the present study is that transplantation antigens were not present at 13 days but developed after the graft was introduced into the ear of the recipient.

Further experiments would be necessary to resolve this possibility.

The graft giant cells

The origin of these cells from either the donor or the recipient must be considered. The evidence strongly favours their origin from trophoblast, inadvertently transplanted with the visceral yolk sac. Firstly pieces of the excised membrane, some of which was grafted, were processed and examined by light microscopy. This revealed the presence of para-placental chorion, containing trophoblast. Secondly, the morphology of the giant cells is similar to that of the obplacental giant cells. The graft giant cells have a diameter of 60 - 80 μm. The obplacental giant cells are 100 - 120 μm in diameter at 16 days post coitum. Both cell types have a fibrillary cytoplasm which stains faintly with haematoxylin and are surrounded by a thick hyaline membrane which stains with PAS reagent and is diastase resistent. Both cell types seem to be migratory and presuming that both are of foetal origin, are able to resist allograft rejection even though in the case of the graft giant cells they may be surrounded by numerous host lymphocytes. Finally at 6 days and 14 days after grafting a similar proportion of grafts excised showed giant cells, suggesting that the giant cells are able to resist allograft rejection and that the introduction of trophoblast into the original graft was a matter of chance.

The trophoblast introduced with the visceral yolk sac may originate from either the paraplacental chorion or the remnant of the bilaminar omphalopleure adjacent to the sinus terminalis.

An immunological classification of foetal membranes

The viviparous conceptus is classically divided into the foetus

and the foetal membranes. The latter is further subdivided into the chorio-allantoic placenta and the accessory foetal membranes which are generally accepted as playing a minor role in the nutrition of the foetus. However, in the rabbit and some other species, the inverted visceral yolk sac is a complex membrane which is extremely important in the nutrition of the foetus.

When considered from an immunological point of view, the accessory foetal membranes are of great importance. The conceptus may be divided into those parts which contain transplantation antigens and those parts which do not. Theoretically, if a part of the conceptus does not make contact with the host, the mother, then it may contain transplantation antigens and yet not be rejected. It is also possible that those parts of the conceptus which contain transplantation antigens are prevented from making contact with the maternal tissues by structural arrangements within the conceptus. These two hypotheses form the basis for an immunological classification of foetal membranes.

Type 1

Foetal membranes which make direct contact with the maternal tissues, and which, if they contain transplantation antigens, may therefore elicit an immune response.

Type 2

Foetal membranes which do not make direct contact with the maternal tissues, but which are not separated from maternal tissues by another foetal membrane.

Type 3

Foetal membranes which are separated from maternal tissues by another foetal membrane and therefore cannot elicit an immunological contact, even if they contain transplantation antigens.

This classification obviously hinges on the definition of direct contact, if we define it as the maximum distance between graft and recipient tissues which will allow sensitisation of the recipient and rejection of the graft we merely become involved in a circular argument, because the maximum distance is unknown as indeed, is the mechanism of sensitisation of the host unsolved. Presumably, if information is to pass between graft and recipient cells to elicit the allograft response, the separation must be of electron microscopic dimensions.

Since there is so little information on the passage of information at the molecular level I have turned to an analogous situation, namely the induction of embryonic cells during morphogenesis. Although, as Lehtonen et al (1975) point out "little is known about substances carrying morphogenetic messages in such interactive processes", three theories have been advanced concerning the passage of information between cells during morphogenesis.

1. Transmission by cell to cell contact.
2. Transmission by diffusion of soluble signal molecules over a space of micrometers.
3. Transmission by interaction of compounds in an extra cellular matrix which is secreted by cells but is not an integral part of those cells.

The term "cell contact" is used by Lehtonen to mean contact between the molecules of the cell surfaces. He also regards cells separated by basal lamina as being in contact. At present the evidence is strongly in favour of the first theory. Cell to cell contact is necessary for morphogenetic interactions. For example, an interspace of 10 - 20 nm between interacting tissue components has been found in the branching rat salivary gland (Culter and Chaudry 1973) and developing rat duodenal mucosa (Mattau, Herniosi and Trian 1972). Lehotonen et al state that by their definition this interspace does not exclude functional cell contacts.

Transplantation antigens are relatively insoluble (Batchelor 1965). Therefore, if one accepts that there is an analogy between morphogenesis and the passage of information in the afferent limb of the allograft response, the maximum distance between graft and host cells in order to allow sensitisation of the host must be approximately 20 nm, and this distance is adopted here as an operational definition of direct contact.

A hypothesis concerning the arrangement of the foetal membranes of the rabbit

During development of the foetal membranes, the only tissue which makes direct contact with the maternal tissues is trophoblast. At nine days the endoderm of the developing visceral yolk sac is separated from the placental folds by a layer of trophoblast, mesoderm and basal lamina. Even if the endodermal cells do contain transplantation antigens at 9 days, they do not make direct contact with the maternal tissues and may therefore be

considered as part of a Type 3 membrane, on the other hand, the chorion makes direct contact with maternal tissues and is classified at Type 1.

The bilaminar omphalopleure on the 9th day consists of an outer layer of trophoblast which is in direct contact with the uterine epithelium, and an inner layer of endoderm which is not in direct contact. There is no information from this study about the presence of transplantation antigens in the endoderm of the bilaminar omphalopleure. The transformation of the uterine epithelium to a symplasma and sloughing of the whole complex at about 10 days might be interpreted as a direct result of such an immunological interaction between cells of the bilaminar omphalopleure and the uterine tissues, but this is entirely speculative.

On the 10th day the visceral yolk sac begins to invert and invaginate into the cavity of the yolk sac. It remains a Type 3 membrane in relation to the antimesometrial uterine wall until the bilaminar omphalopleure (parietal layer of yolk sac) breakes down at the end of the 10th day. On the 13th day the visceral yolk sac is a Type 2 membrane related to, but not in direct contact with, the antimesometrial wall. The Type 1 membranes at day 13 are the chorio-allantoic placenta, the paraplacental chorion and the remnant of the bilaminar omphalopleure which is related to the sinus terminalis.

In a teleological sense there appears to be a set of accessory foetal membranes which prevent the visceral yolk sac from making direct contact with the maternal tissues. Thus, the visceral yolk sac as it inverts is protected from the antimesometrial uterine

wall by the bilaminar omphalopleure. After the visceral yolk sac has inverted, the bilaminar omphalopleure breaks down leaving a space between the visceral yolk sac and the uterine epithelium. At the periphery of the visceral yolk sac the bilaminar omphalopleure remains intact, thereby preventing any direct contact between the bulging endoderm covering the sinus terminalis and the uterine epithelium.

The visceral yolk sac is anchored to the paraplacental fissure by the paraplacental chorion. Sansom (1927) observed that trophoblast sloughs off the paraplacental chorion and invades the endometrium and trophoblast sloughs into the paraplacental fissure. He suggested that this may occur "possibly with the object of reducing the area of attachment of the placental disc to the uterine wall preparatory to parturition". This marked activity occurs about the 17th day which is well before parturition. I suggest that this activity of the paraplacental chorion prevents overgrowth of the endometrium of the periplacental fissure and prevents direct contact occurring between the visceral yolk sac and the maternal tissues.

Direct contact occurs when the trophoblastic knobs invade the uterine epithelium at about the 7th day. Before this time the blastocyst has been prevented from making direct contact with the maternal tissues by the zona pellucida. The trophoblast knobs burst through the zona pellucida and make cell to cell contacts with the uterine epithelium (Steer 1969, 1971, 1971b). The zona pellucida disappears soon after trophoblast knob invasion. The trophoblast knobs eventually penetrate the maternal blood vessels. There is no experimental evidence to suggest that

trophoblast knob invasion is involved in immunological protection of the conceptus. The invading trophoblast is not rejected by the mother.

The obplacental giant cells probably originate from the invading trophoblast of the trophoblast knob. Again these giant cells are not rejected by the mother even though they invade the myometrium which contains lymphatics. The trophoblast which invades maternal tissues undergoes a morphological change. The cells enlarge greatly, the cytoplasm differs in its staining properties and each giant cell is surrounded by hyaline membrane. Gardner et al (1973) has suggested that giant cell transformation of the trophoblast cells is the normal path of differentiation so long as the trophoblast cells are not in contact with the inner cell mass. However, there does remain the possibility that the transformation of trophoblast is a direct result of implantation into an alien environment and that this transformation protects the trophoblast from an allograft rejection. It would be interesting to know whether obplacental giant cells occur in pure bred rabbit matings. Jones and Kemp (1969) have suggested that trophoblast coats its cell surface with a hyaline membrane which is specific to a particular host. This cell coat then protects the trophoblast from an allograft rejection response. They suggest that this specificity of cell coat is determined by the passage of messenger molecules, possibly RNA from the maternal cells to the trophoblast which is then able to manufacture the specific cell coat. This theory is at present entirely speculative, but if it is true then trophoblast knob invasion may be involved in the passage of this information.

Summary

Aims of the study

These were to determine the presence or absence of transplantation antigens on the visceral yolk sac of the rabbit and using the morphological study, to provide a hypothesis as to how the membrane escapes rejection by a maternal allograft response.

Results of the morphological study

New observations from the morphological study provide a greater understanding of the implantation site of the rabbit uterus. The development and relationships of the foetal membranes are described in greater detail than before.

Results of the grafting experiments

Evidence is presented that the visceral yolk sac of the rabbit contains transplantation antigens at 13 days. The trophoblast of the paraplacental chorion and/or bilaminar omphalopleure resists the allograft rejection response which has destroyed the endodermal cells of the visceral yolk sac.

Conclusions

A hypothesis is presented to account for the survival of the visceral yolk sac in situ despite the apparent presence of transplantation antigens on the endodermal cells. The discussion goes on to speculate on the function of the paraplacental chorion, the bilaminar omphalopleure and the trophoblastic knobs from an immunological point of view.

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PHOTOMICROGRAPHS OF THE MORPHOLOGICAL STUDY

Fig. 1 THE NON-PREGNANT UTERUS (LENGTH - 38 mm)

The uterus has been opened along the antimesometrial border. The placental folds are shown. They are divided into separate coussinets.

P - Placental fold

C - Coussinet

Fig. 2 A CROSS-SECTION OF THE NON-PREGNANT UTERUS

METHYLENE BLUE x 6.3

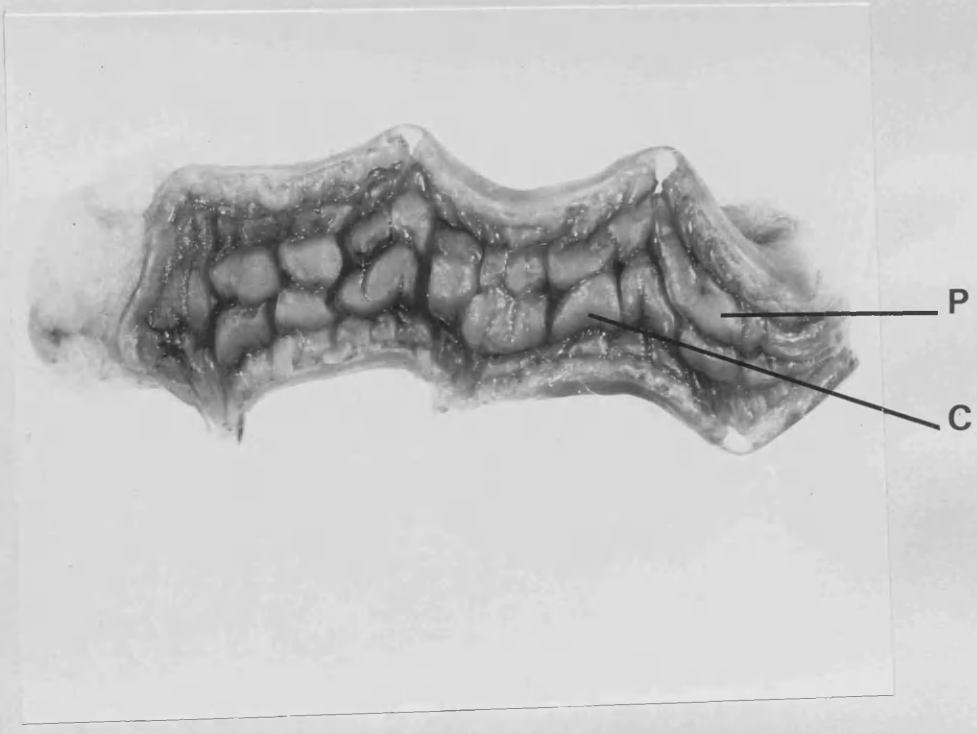
The non-pregnant uterus has been sectioned to show the configuration of the uterine folds.

P - Placental fold

UL - Uterine lumen

M - Myometrium

Ms - Mesometrium


P
C

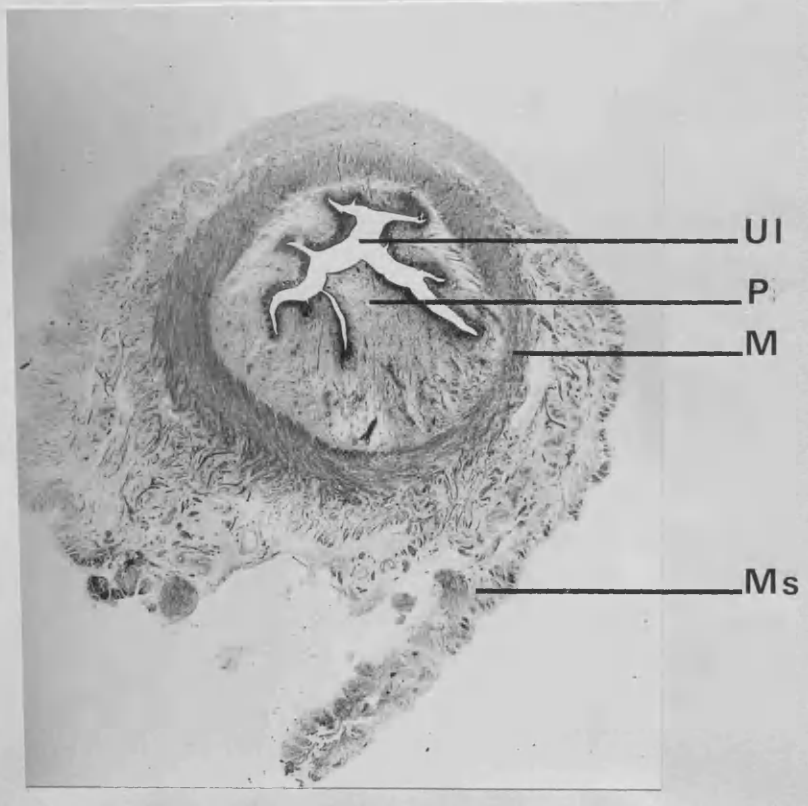

UI
P
M
Ms

FIG. 3. THE EPITHELIUM LINING THE INTER-PLACENTAL FISSURE (Methylene blue x 250)

Uterine epithelium is made up of simple columnar cells which occasionally bear microvilli at this particular part of the uterus.

FIG. 4. SEM OF THE NON-PREGNANT PLACENTAL AND PERIPLACENTAL FOLDS. (x 20).

The uterus has been opened along the antimesometrial border and shows in the upper part the placental folds. In the lower part of the picture the periplacental fold can be seen.

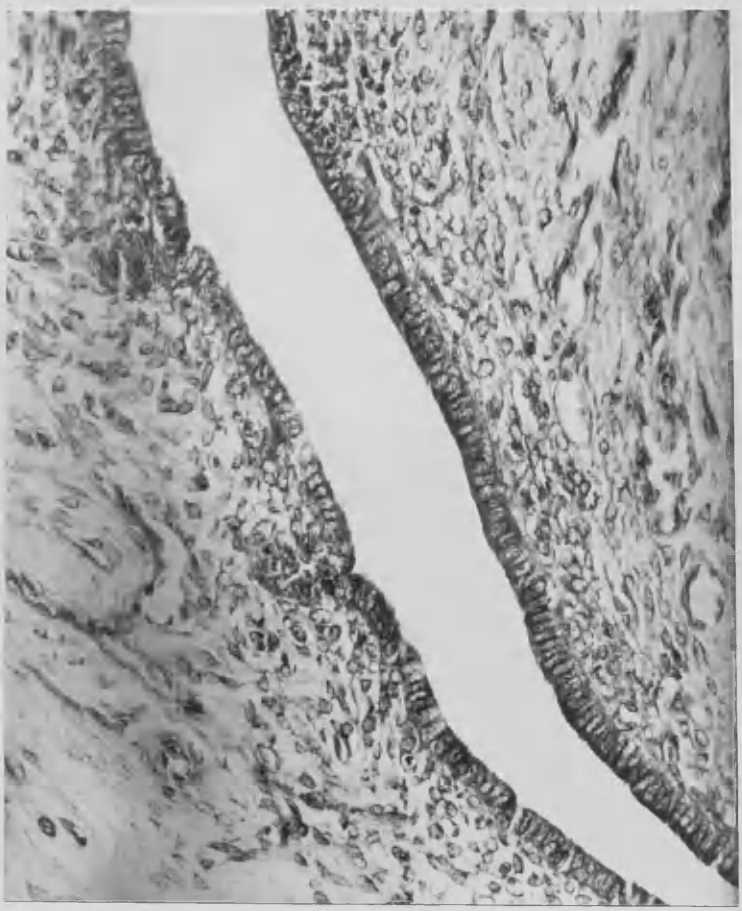


Fig. 5 SEM OF THE OPENING OF A SIMPLE TUBULAR MUCUS SECRETING GLAND OF THE UTERINE EPITHELIUM

x 100

Note the many ciliated cells which surround this opening.

Fig. 6 TRANSVERSE SECTION OF THE 7 DAY CONCEPTUS

H & E x 17

The conceptus has been sectioned transversely.

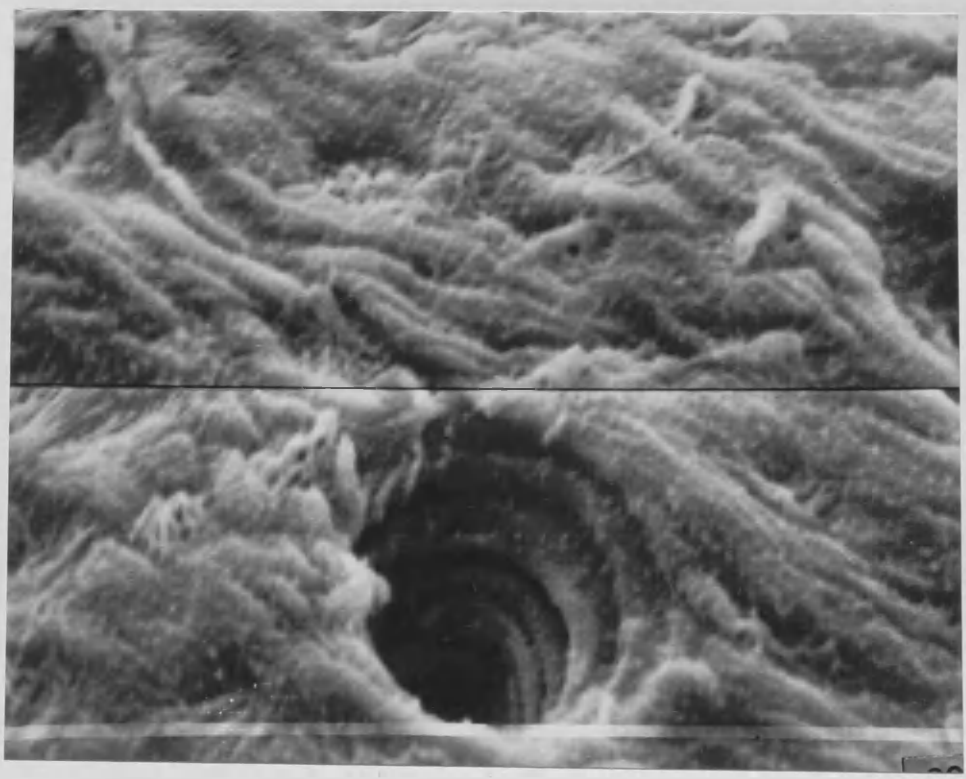
The placental folds, antimesometrial wall and developing blastocyst are shown.

Am - Antimesometrial wall

B - Blastocyst

P - Placental folds

Ms - Mesometrium



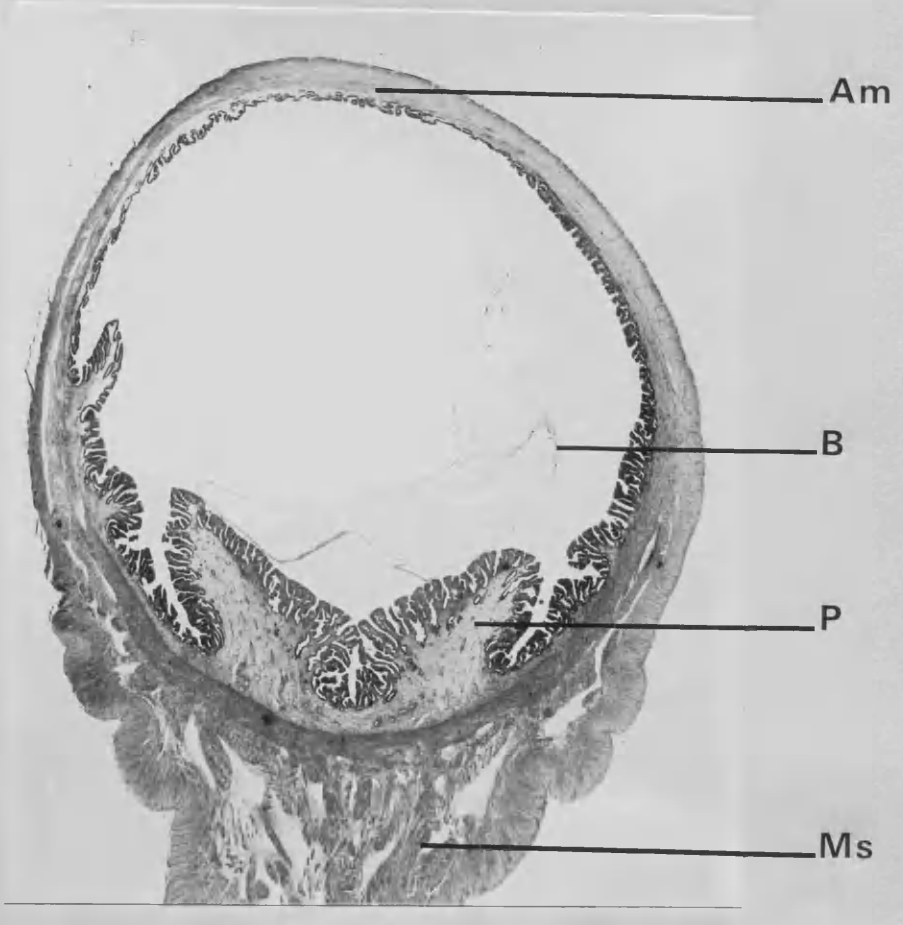

Am
B
P
Ms

FIG. 7. SEM OF THE PLACENTAL FOLDS AT 7 DAYS (x 10).

The antimesometrial wall has been removed to reveal the placental folds forming a cup for the blastocyst which has been removed.

FIG. 8. THE RELATIONSHIPS BETWEEN THE PLACENTAL FOLDS AND BLASTOCYST AT 7 DAYS (PAS x 63).

The uterine epithelium is separated from the developing blastocyst by the zona pellucida, which stains darkly with PAS.

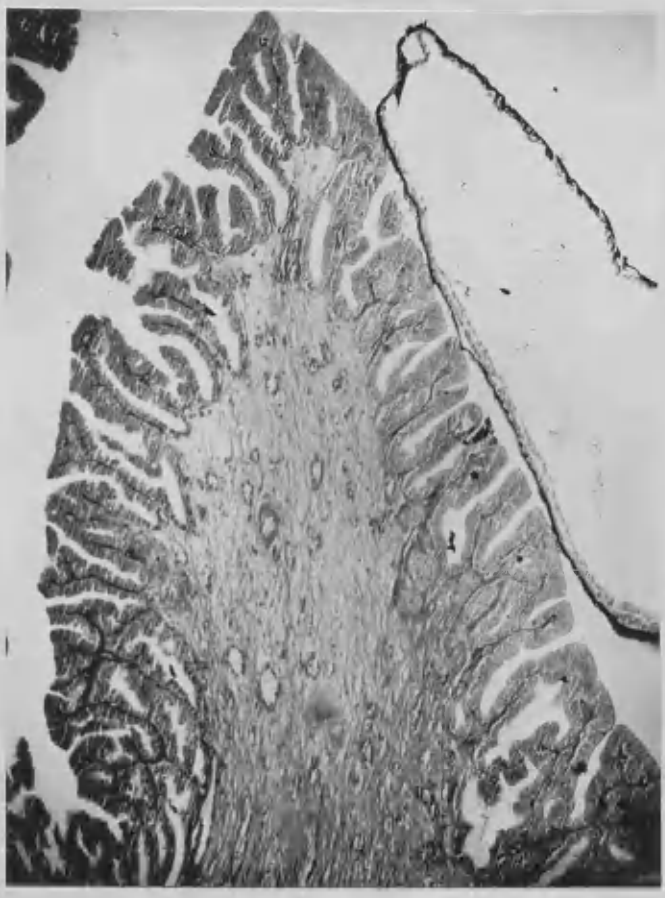
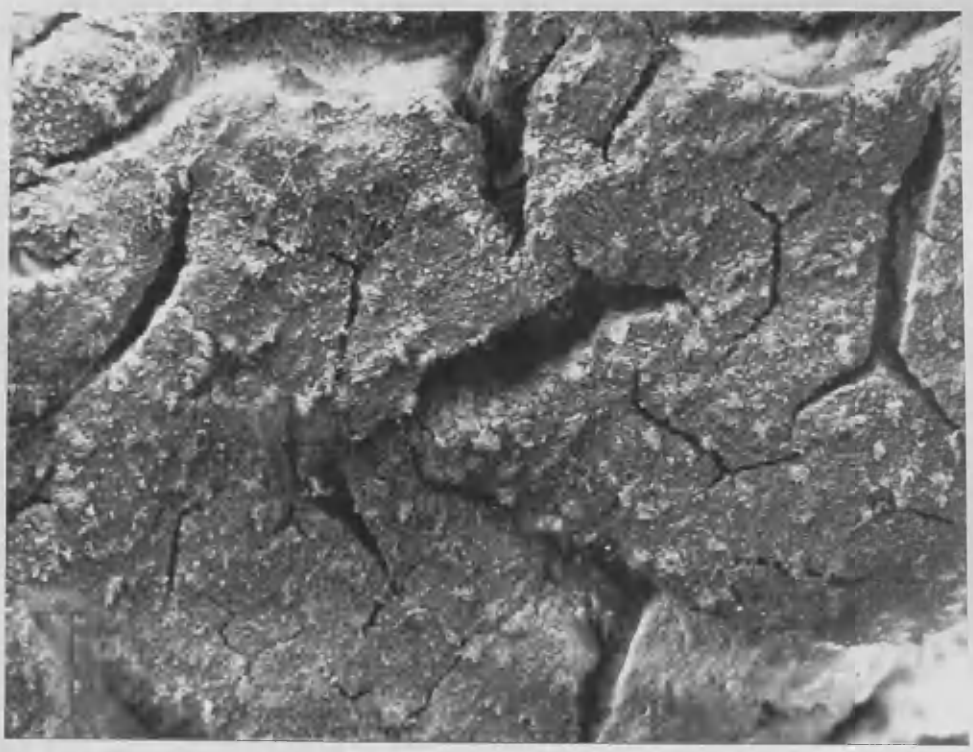


FIG. 9. SEM OF THE PLACENTAL FOLDS AT 7 DAYS (x 200). Numerous fissures are present, some of these appear to be artifacts caused by processing of the sample. Note the numerous cilia.

FIG. 10. SEM OF THE PLACENTAL FOLDS AT 7 DAYS (x 2,000). The apices of the uterine epithelial cells are covered by microvilli. Four ciliated cells are shown.



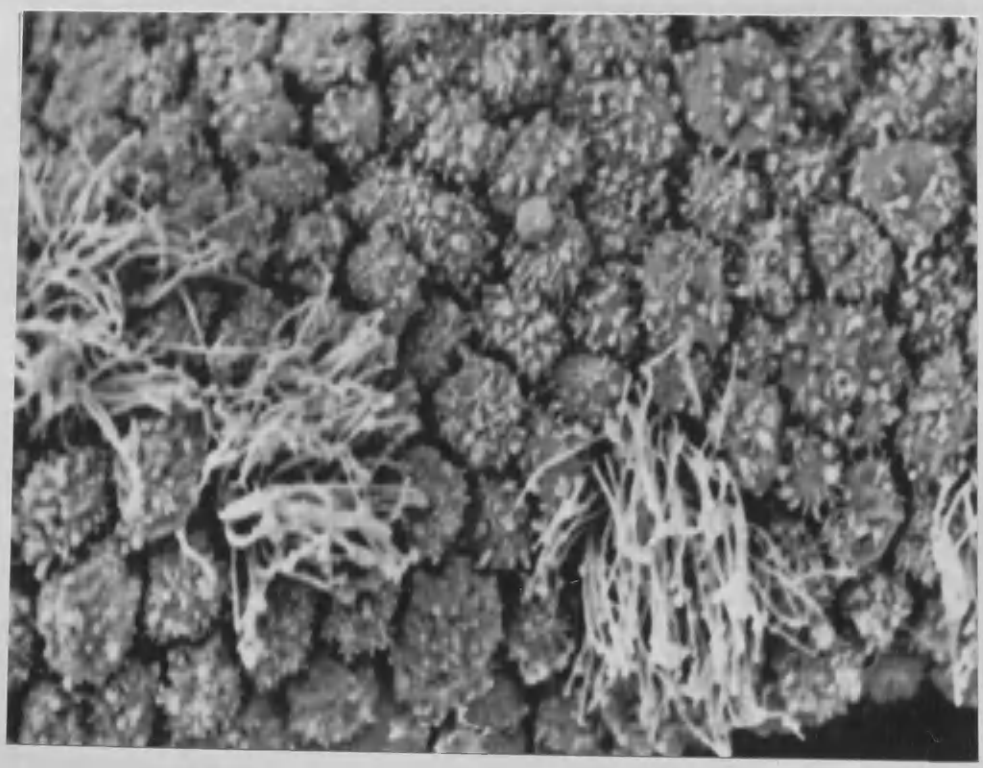


Fig. 11 THE RELATIONSHIPS BETWEEN THE EMBRYO AND SURROUNDING MEMBRANES AT 9 DAYS

H & E x 20

Compare with [redacted]

Em - Embryo

P - Placental fold

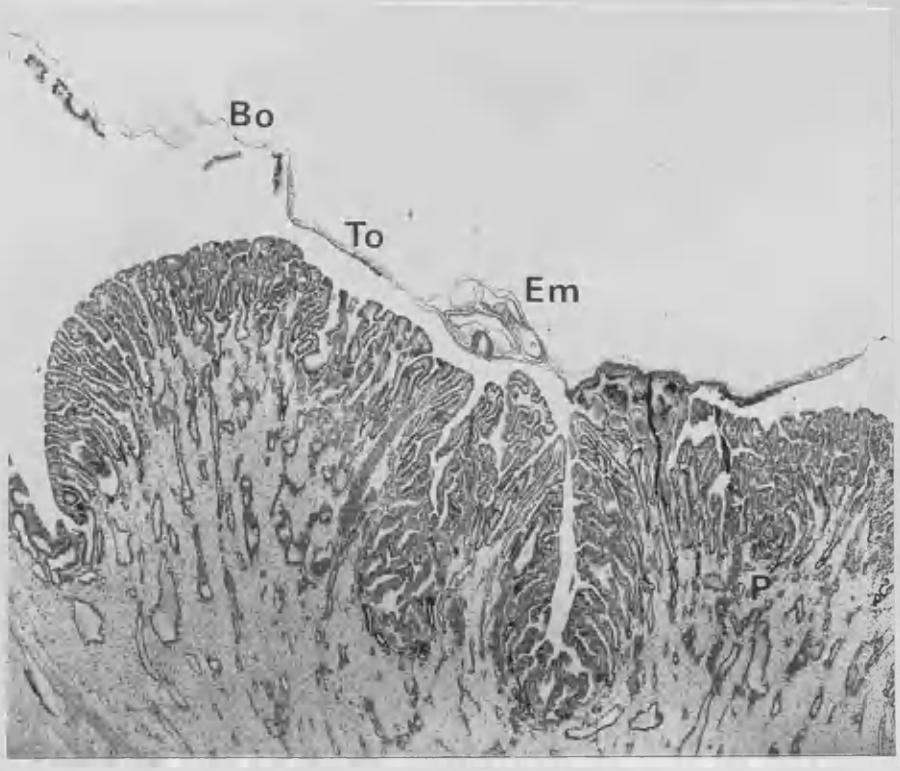
To - Trilaminar omphalopleure

Bo - Bilaminar omphalopleure

Fig. 12 THE INVADING TROPHOBLAST AND OVERLYING DEVELOPING VISCERAL YOLK SAC

H & E x 100

A detail of Fig. 11.


Bo
To
Em
P

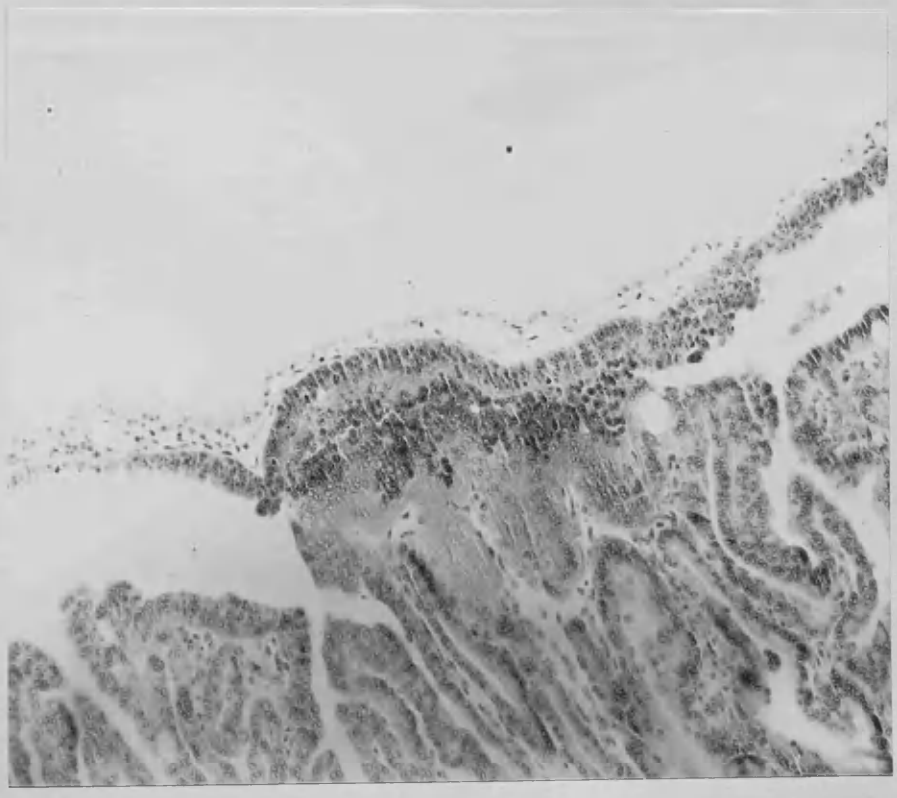


Fig. 13 THE DEVELOPING VISCERAL YOLK SAC AND CHORION OVERLYING A PLACENTAL FOLD AT 9 DAYS

H & E x 335

A detail of Fig. 11.

E - Endoderm

Vv - Vitelline vessel containing foetal blood cells

C - Chorion

P - Placental fold

Fig. 14 THE RELATIONSHIPS BETWEEN THE EMBRYO, FOETAL MEMBRANES AND MATERNAL TISSUES AT 10 DAYS

H & E x 20

Compare with [redacted]

Em - Embryo

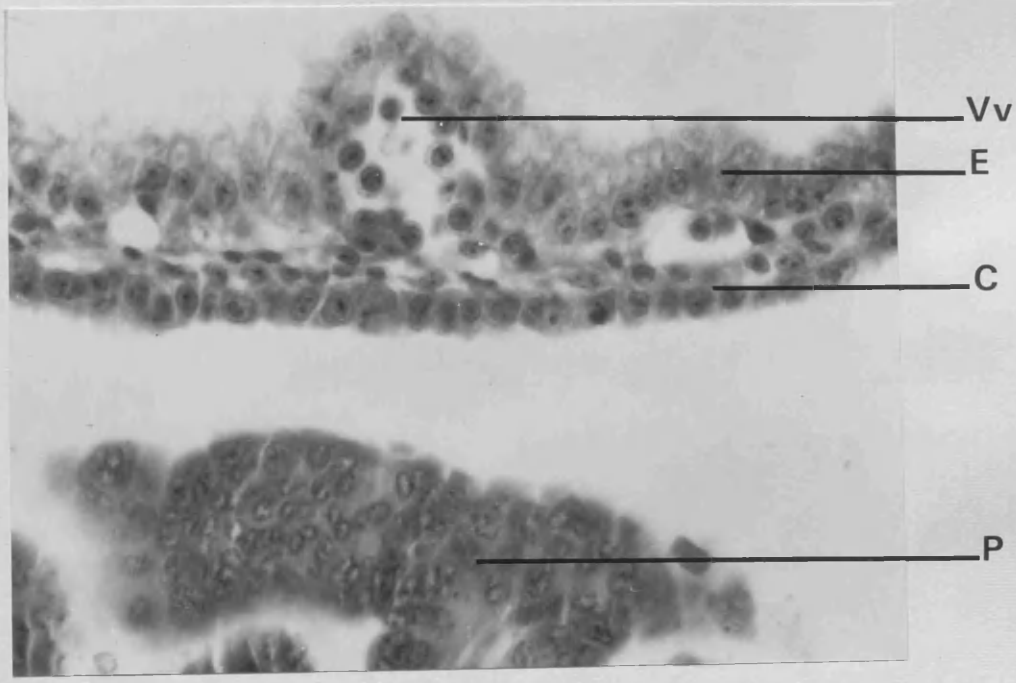
To - Trilaminar omphalopleure

Vy - Visceral yolk sac

P - Placental fold

St - Sinus terminalis

Bo - Bilaminar omphalopleure


Vv
E
C
P

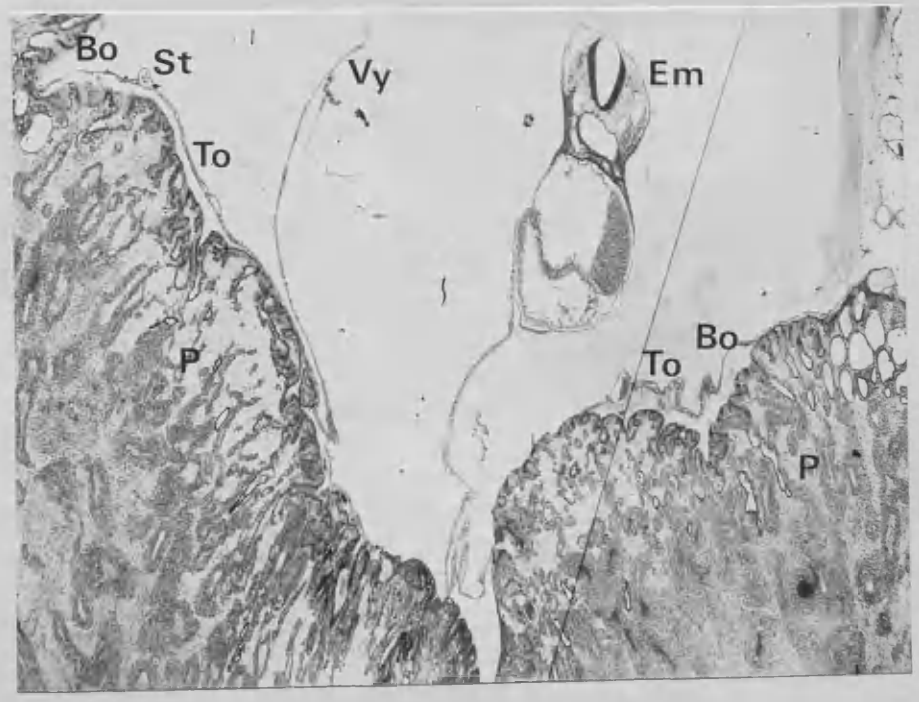

Bo
St
To
P
Vy
Em
Bo
To
P

Fig. 15 THE RELATIONSHIPS BETWEEN THE BILAMINAR OMPHALOPLEURE, SINUS TERMINALIS, TRILAMINAR OMPHALOPLEURE AND THE UTERINE TISSUES AT 10 DAYS

H & E x 100

Detail of Fig. 14.

To - Trilaminar omphalopleure

St - Sinus terminalis

Bo - Bilaminar omphalopleure

Us - Uterine symplasma

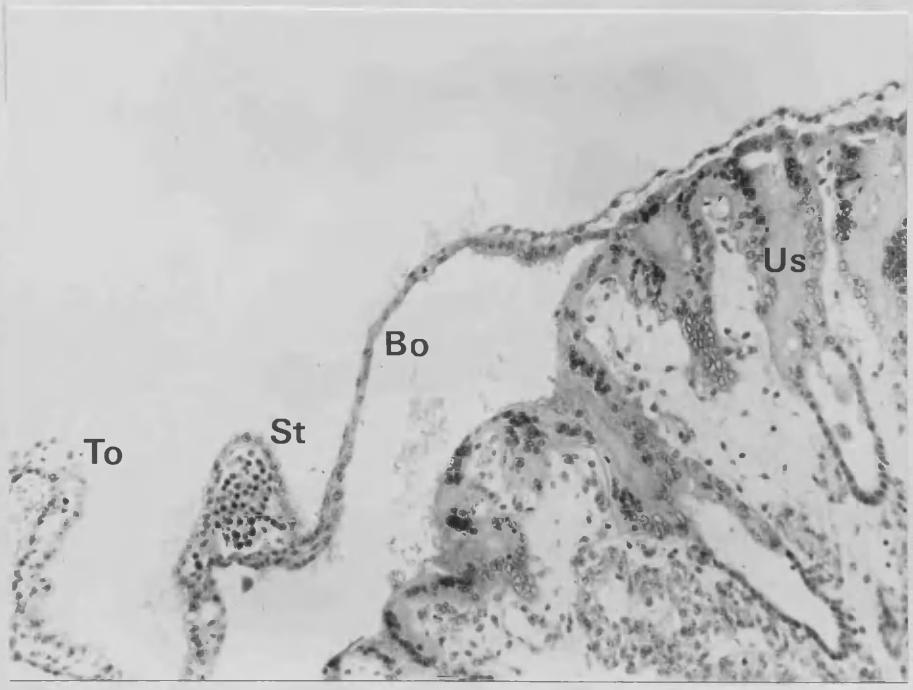
Fig. 16 THE RELATIONSHIPS BETWEEN THE DEVELOPING VISCERAL YOLK SAC, SINUS TERMINALIS AND THE UTERINE TISSUES AT 10 DAYS

H & E x 100

Detail of Fig. 14.

St - Sinus terminalis

Vv - Vitelline vessel


Us
Bo
St
To

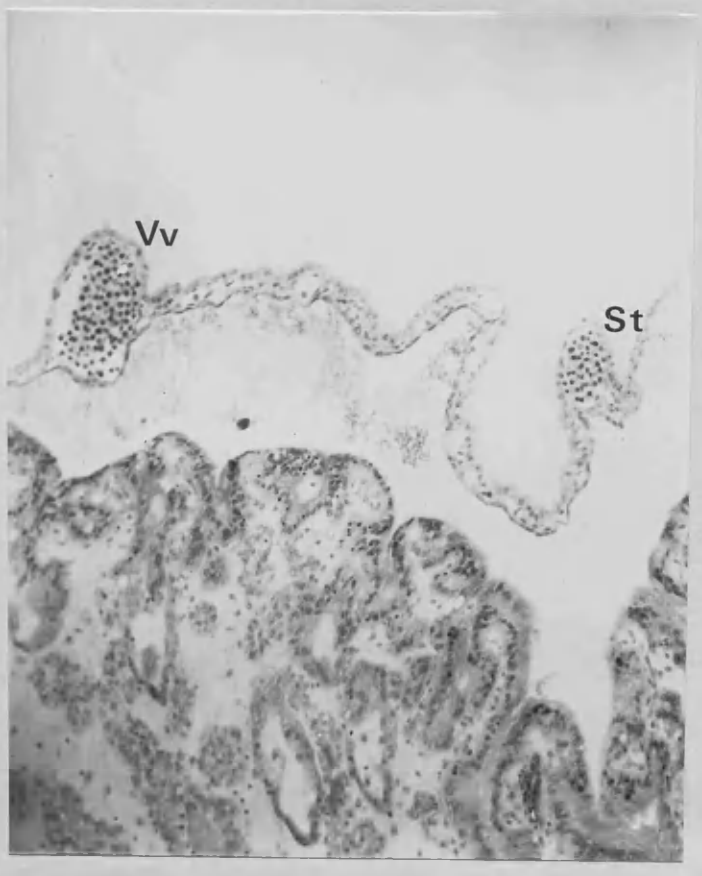

Vv
St

Fig. 17 TRANSVERSE SECTION OF THE RABBIT CONCEPTUS AT 13 DAYS

INCIDENT LIGHT

Compare with 

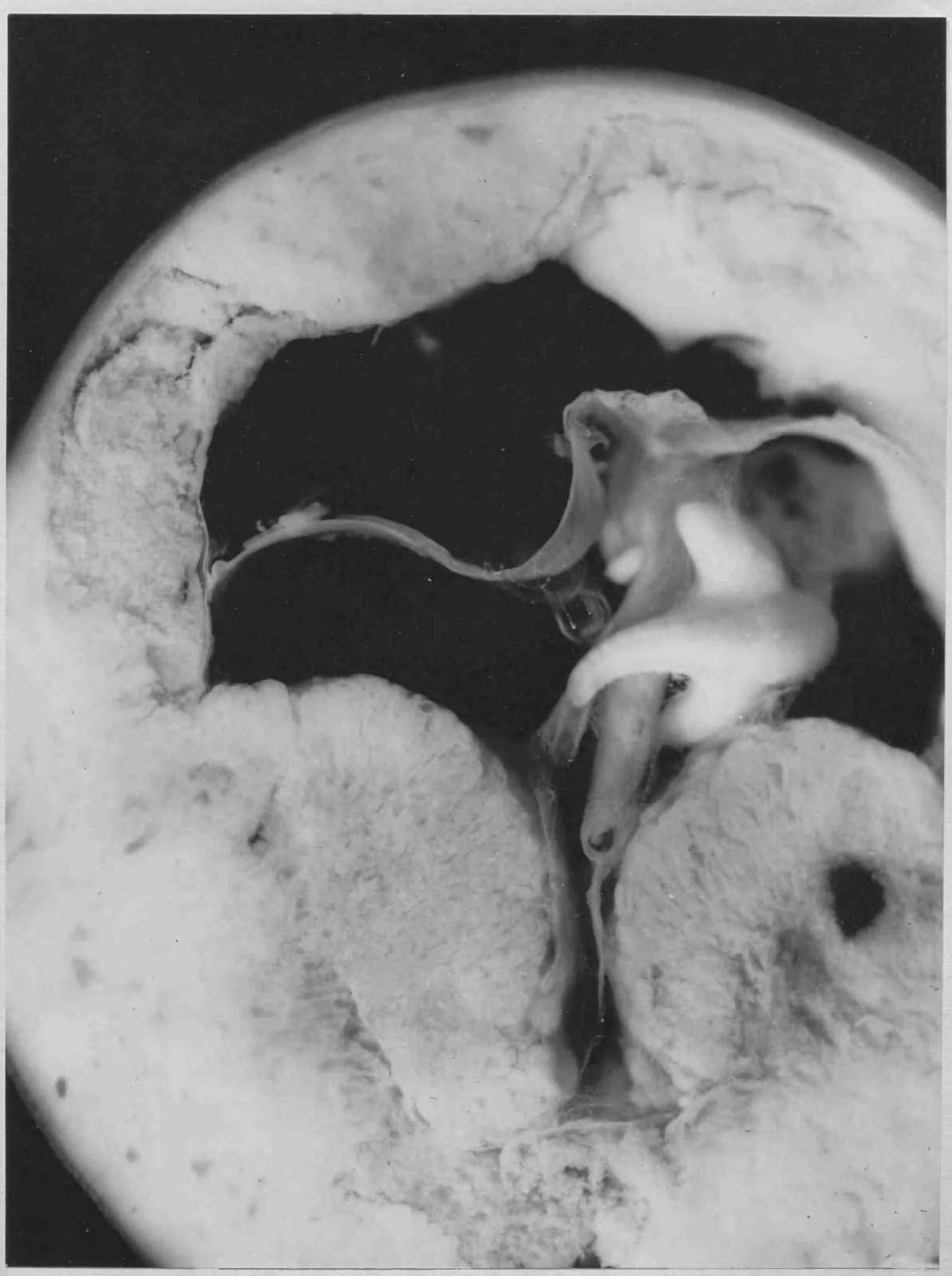


Fig. 18 TRANSVERSE SECTION OF THE RABBIT CONCEPTUS AT 13 DAYS

H & E x 6

Compare with [redacted]

F - Foetus

Vy - Visceral yolk sac ,

Pc - Paraplacental chorion

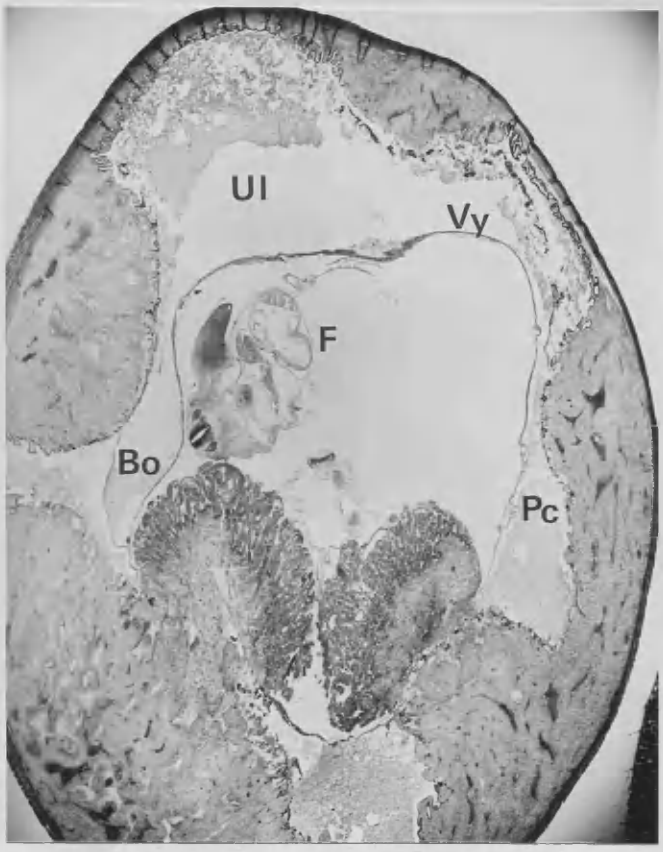
Bo - Bilaminar omphalopleure

Ul - Uterine lumen

Fig. 19 THE RELATIONSHIPS BETWEEN THE VISCERAL YOLK SAC AND THE FOETUS AT 13 DAYS

Incident light x 6

The conceptus has been opened along the antimesometrial wall by a cruciate incision to reveal the visceral yolk sac through which there are vitelline vessels coursing. The embryo is covered by the visceral yolk sac and amnion.


Ul
Vy
F
Bo
Pc

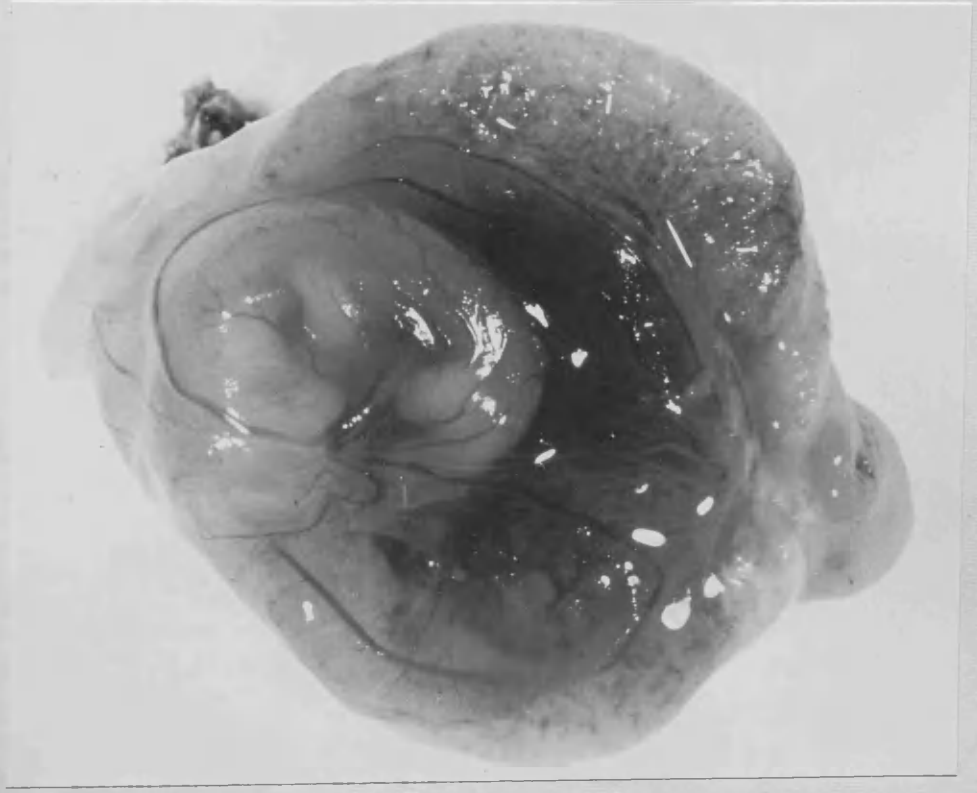


Fig. 20 THE RELATIONSHIPS BETWEEN THE EMBRYO, AMNION AND VISCERAL YOLK SAC AT 13 DAYS

H & E x 250

Detail of Fig. 18.

Vy - Visceral yolk sac

A - Amnion

Ul - Uterine lumen

Vv - Vitelline vessel

Fig. 21 THE RELATIONSHIPS BETWEEN THE VISCERAL YOLK SAC, SINUS TERMINALIS, PARAPLACENTAL CHORION, BILAMINAR OMPHALOPLEURE REMNANT AND THE UTERINE TISSUES

H & E x 100

Detail of Fig. 18.

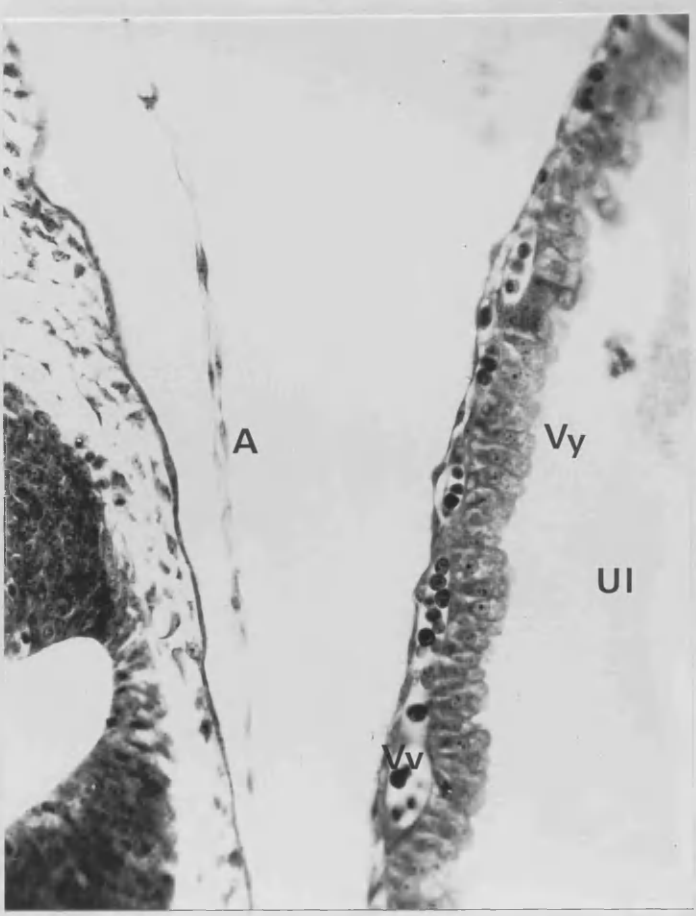
Vy - Visceral yolk sac

St - Sinus terminalis

Bo - Bilaminar omphalopleure

Pc - Paraplacental chorion

Ut - Uterine tissues


A
Vy
Ul
Vv

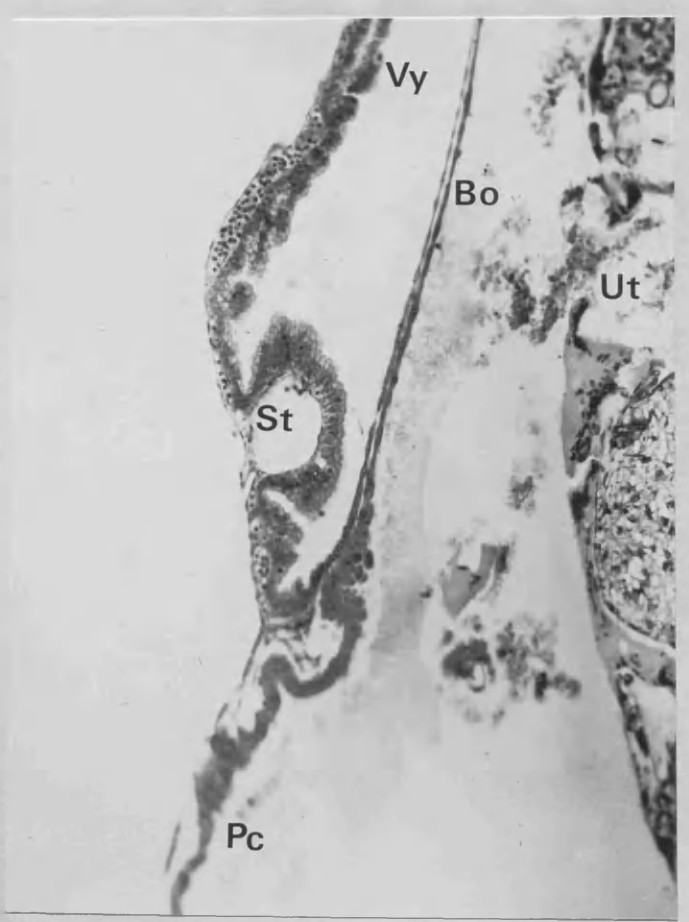
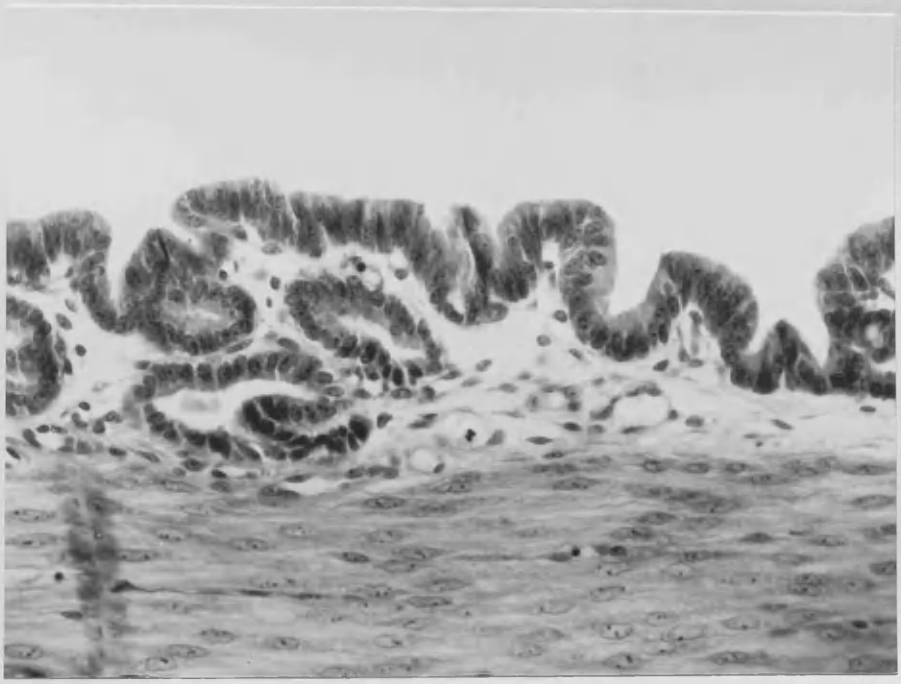

Vy
Bo
Ut
St
Pc

FIG. 22. THE 7 DAY ANTIMESOMETRIAL WALL (H & E x 250).

The blastocyst has shrunk away from the antimesometrial wall.

FIG. 23. SEM OF THE SURFACE OF THE 7 DAY ANTIMESOMETRIAL WALL (x 100).



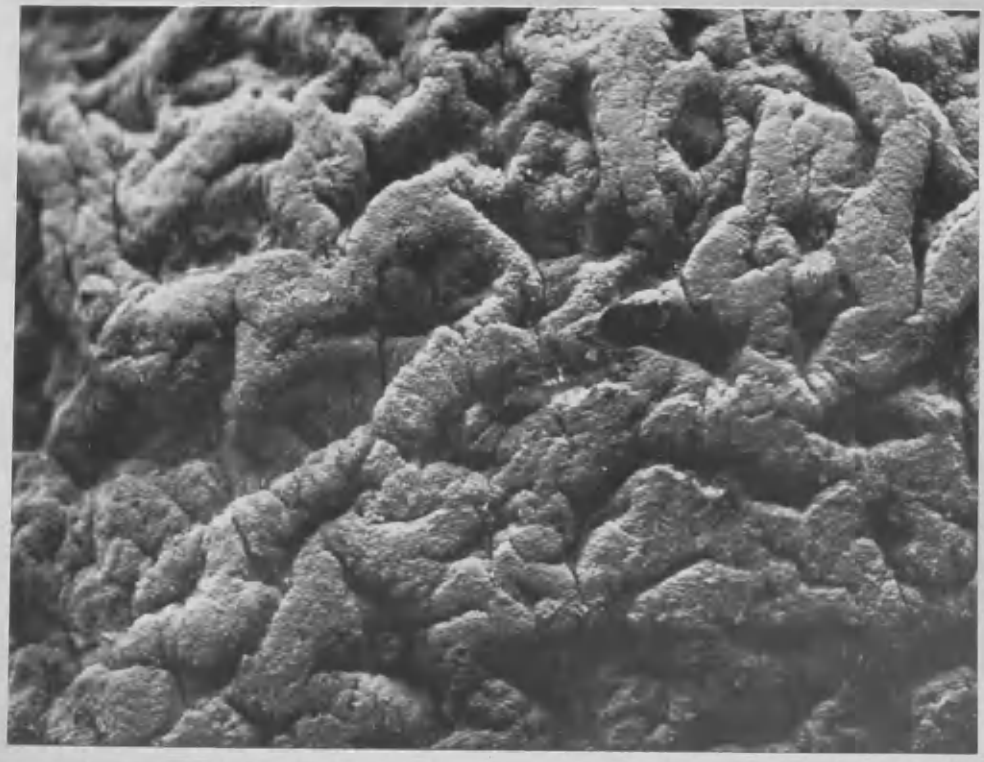
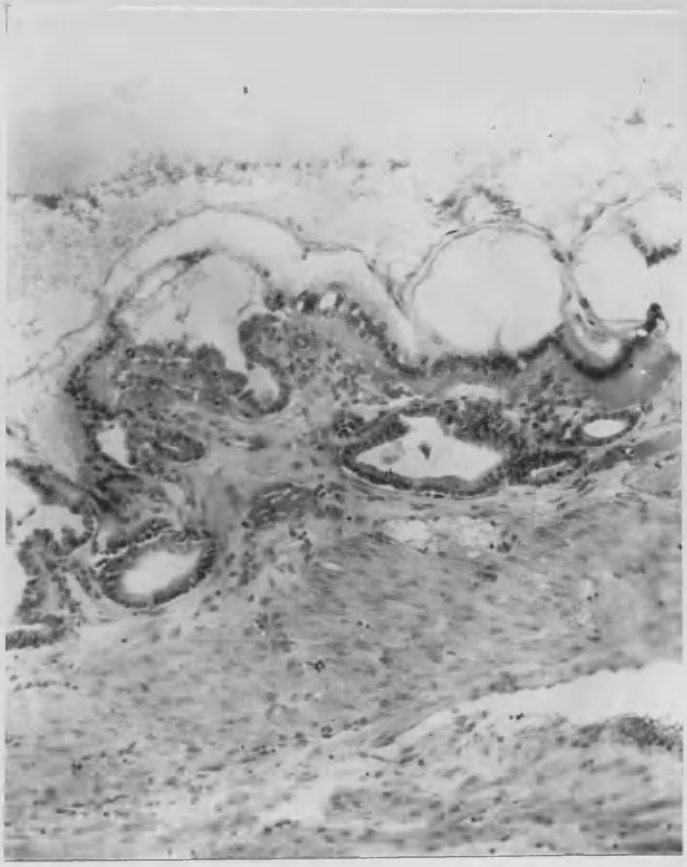


FIG. 24. THE RELATIONSHIPS BETWEEN THE FOETAL TISSUES AND THE UTERINE TISSUES AT 9 DAYS (PAS x 100).

FIG. 25. RELATIONSHIPS BETWEEN THE FOETAL AND UTERINE TISSUES AT 10 DAYS AT THE ANTIMESOMETRIAL WALL (H & E x 82).



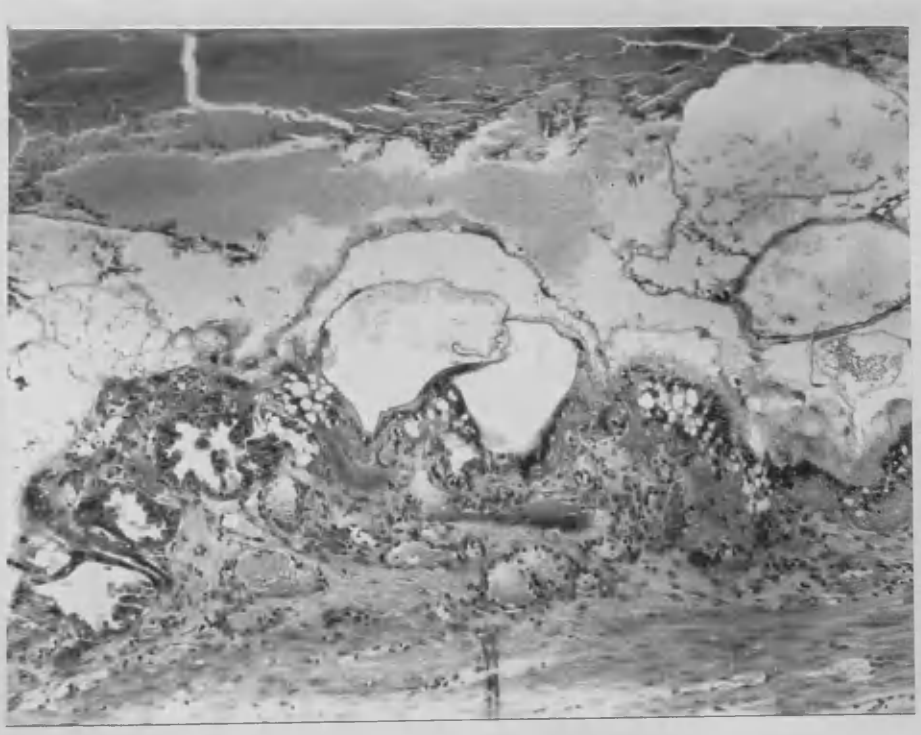


FIG. 26. RELATIONSHIPS BETWEEN THE ANTIMESOMETRIAL WALL AND THE FOETAL TISSUES AT 13 DAYS (Methylene blue x 250).

FIG. 27. SEM OF THE ANTIMESOMETRIAL UTERINE WALL AT 13 DAYS (x 200).

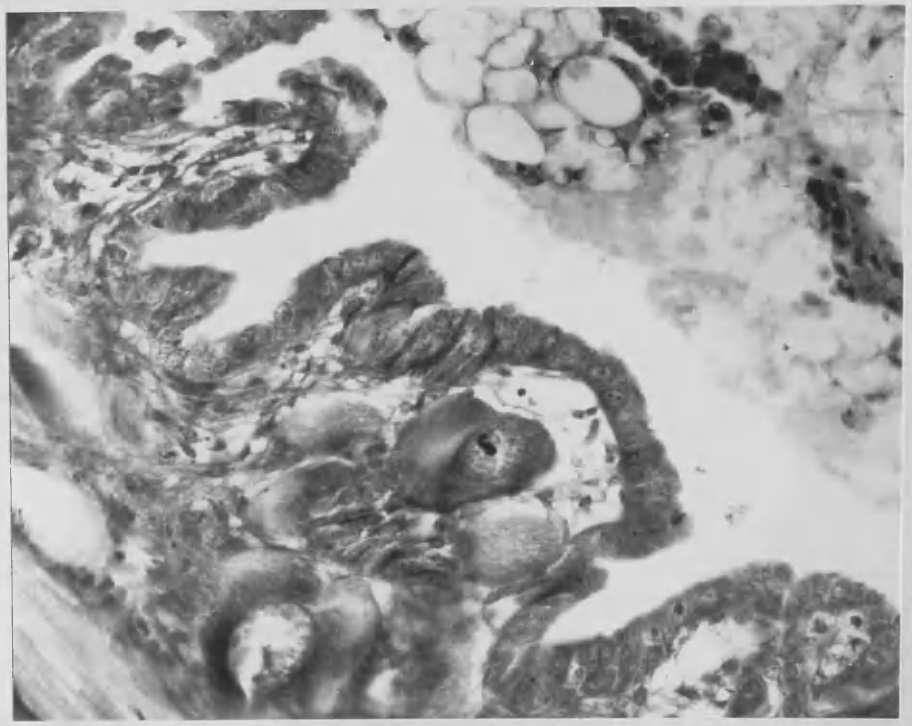
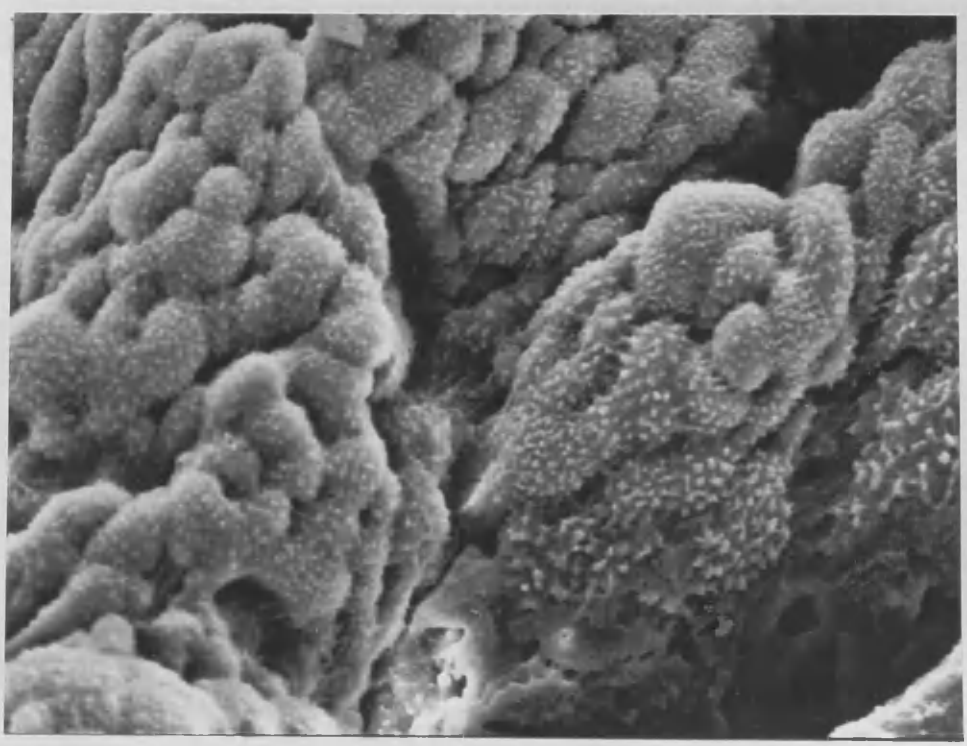


FIG. 28. SEM OF THE ANTIMESOMETRIAL WALL AT 13 DAYS (x 2,000).

FIG. 29. SEM OF THE ENDODERMAL SURFACE OF THE VISCERAL YOLK SAC AT 13 DAYS (x 2,000).



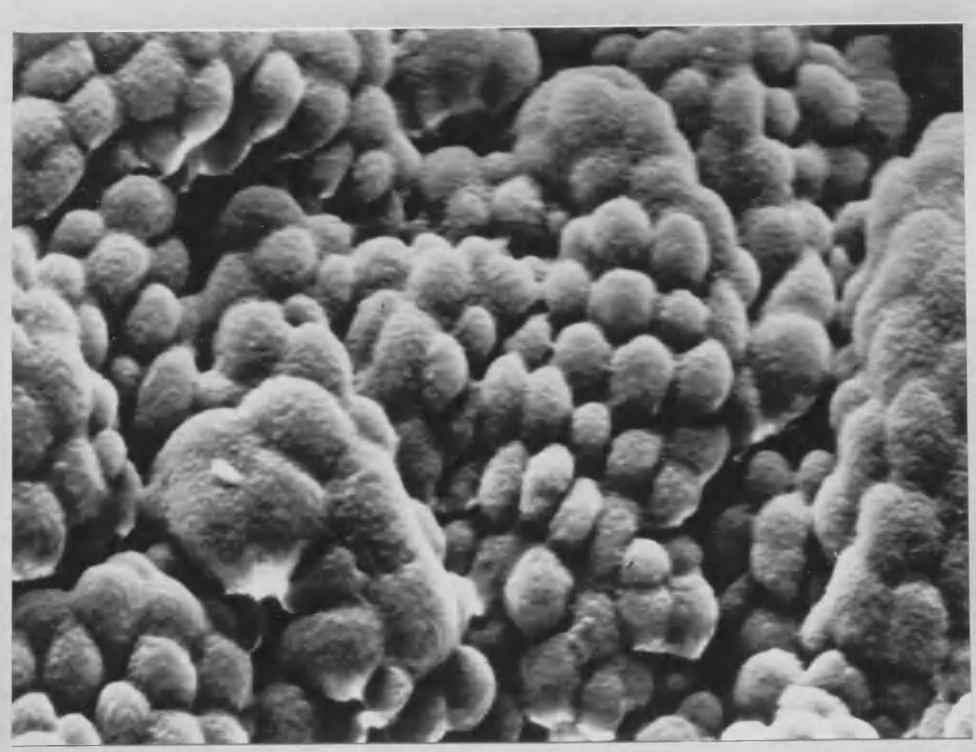


FIG. 30. SEM OF THE ENDODERMAL SURFACE OF THE VISCERAL YOLK SAC AT 13 DAYS (x 5,000).

FIG. 31. SEM OF THE MESODERMAL VISCERAL YOLK SAC SURFACE AT 13 DAYS (x 200).

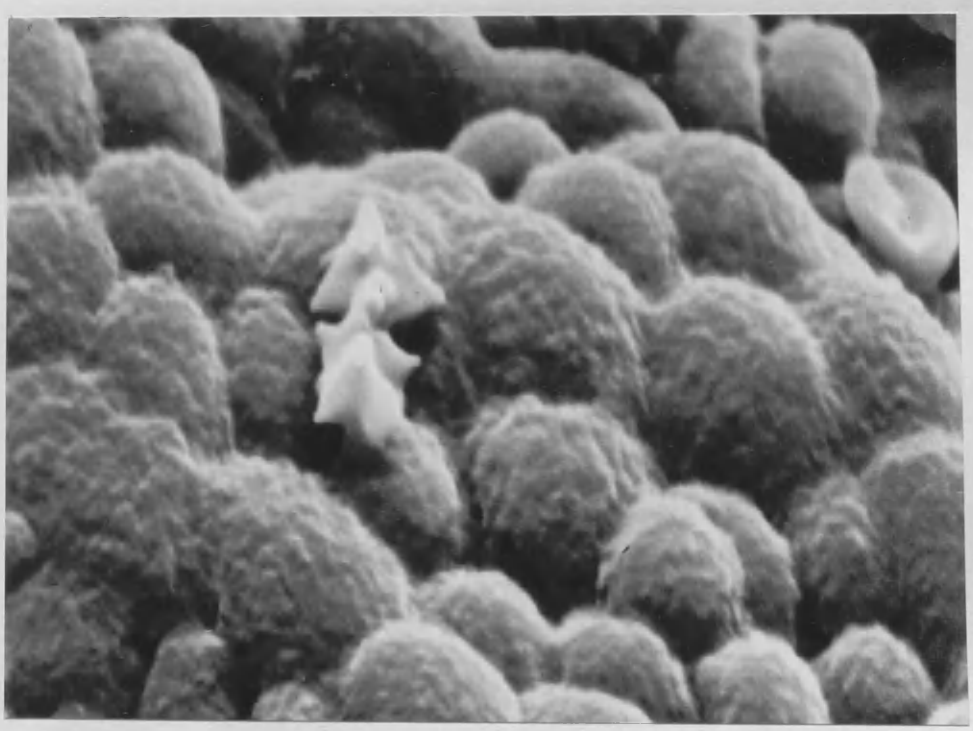
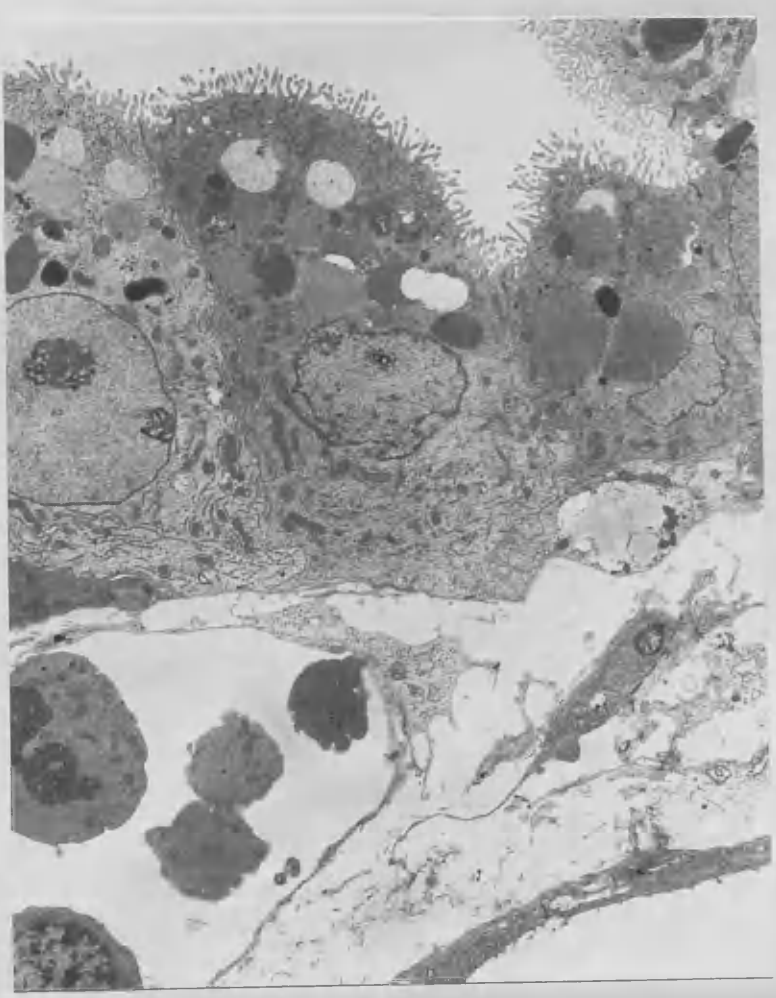


FIG. 32. TRANSMISSION ELECTRON MICROGRAPH OF THE VISCERAL YOLK SAC AT 13 DAYS (x 3,600).

FIG. 33. TRANSMISSION ELECTRON MICROGRAPH OF AN ENDODERMAL CELL OF THE VISCERAL YOLK SAC AT 13 DAYS (x 5,900).



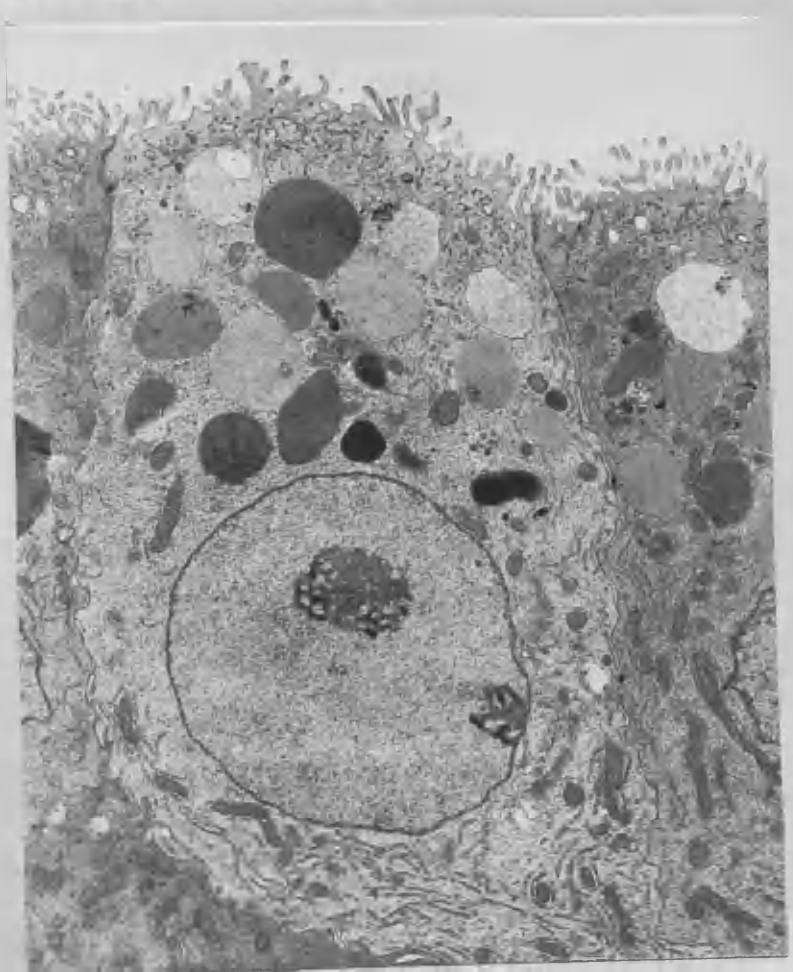
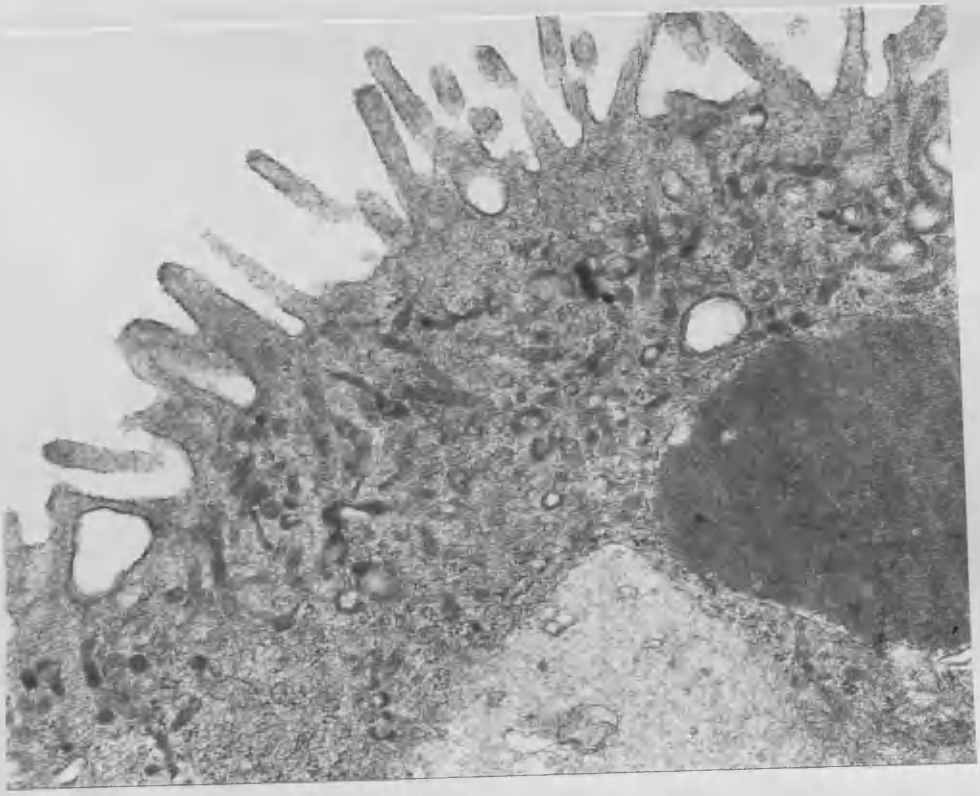


FIG. 34. TRANSMISSION ELECTRON MICROGRAPH OF THE SURFACE OF AN ENDODERMAL CELL AT 13 DAYS (x23,000)

FIG. 35. TRANSMISSION ELECTRON MICROGRAPH TO SHOW MYTOTIC ACTIVITY OF THE ENDODERMAL CELLS OF THE 13 DAY YOLK SAC (x 3,600).



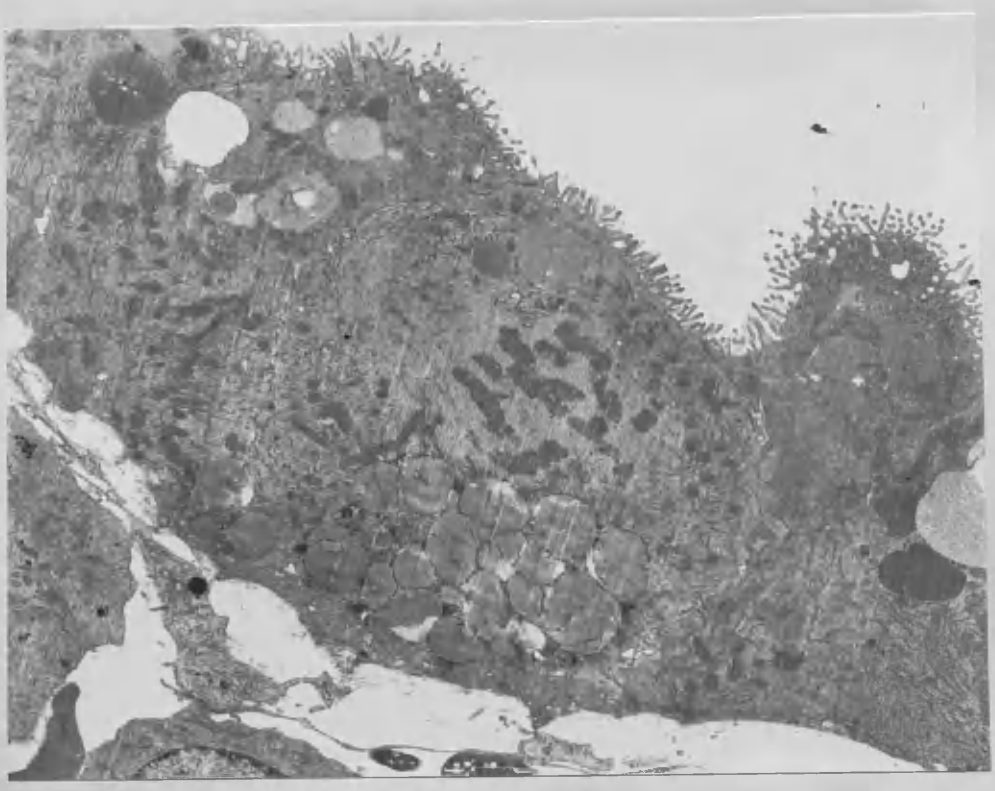
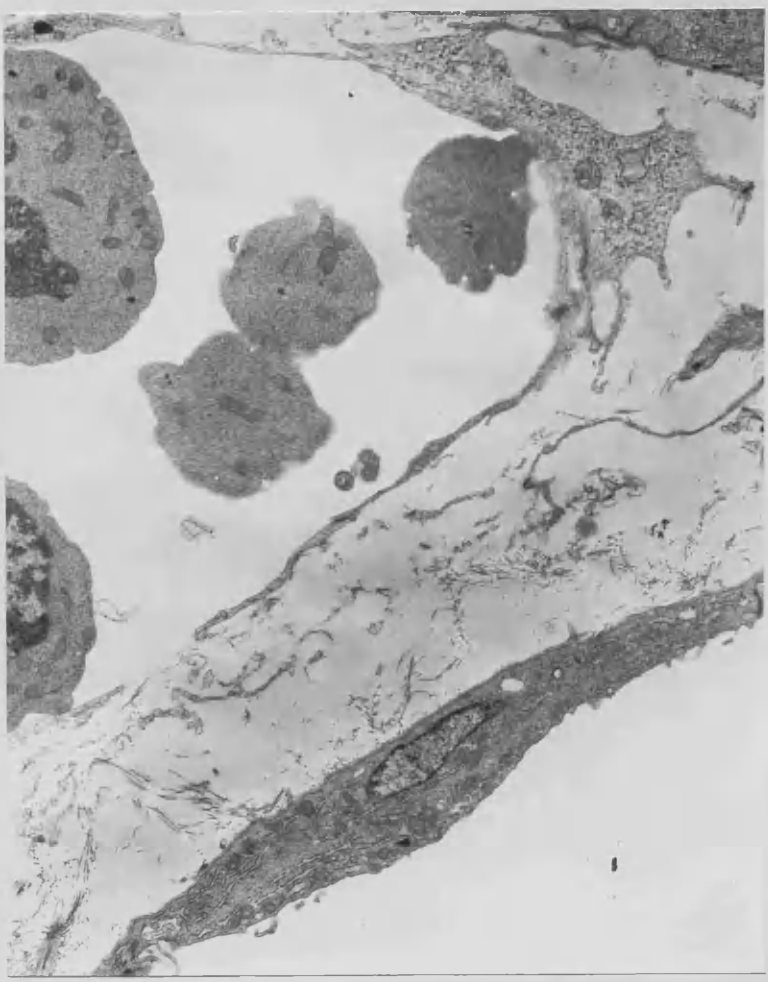
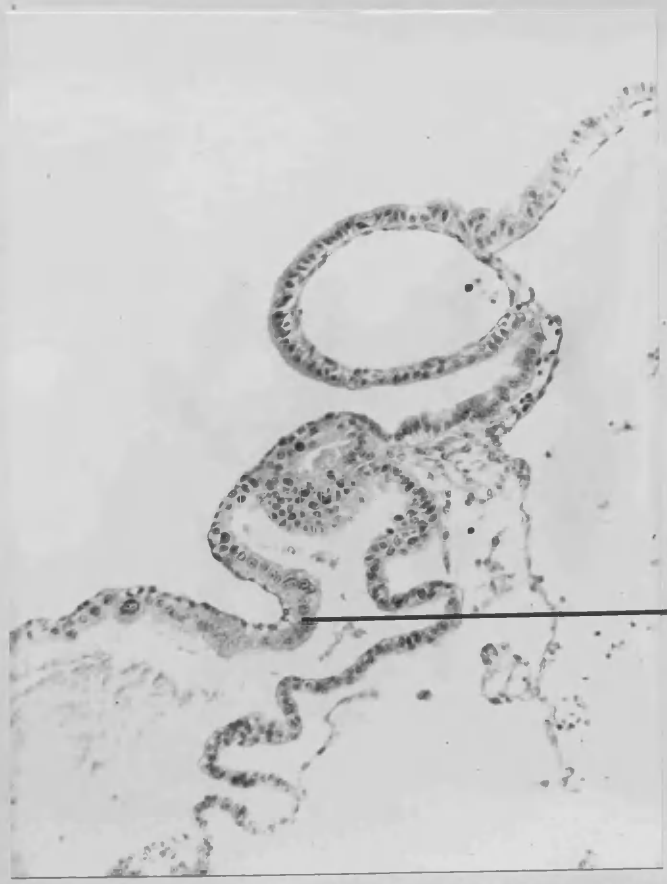


FIG. 36. TRANSMISSION ELECTRON MICROGRAPH OF THE 13 DAY VISCERAL YOLK SAC TO SHOW A VITELLINE VESSEL AND THE MESODERMAL SURFACE OF THE VISCERAL YOLK SAC (x 5,900).

FIG. 37. THE TISSUE WHICH WAS TRANSPLANTED (H & E x 130). This piece of excised visceral yolk sac had been in T199 medium for $\frac{3}{4}$ hour. The columnar endodermal cells are intact and it is noted that paraplacental chorion is also present.





Paraplacental
Chorion

PHOTOMICROGRAPHS OF THE GRAFTING STUDY

A Key to the labels in these photomicrographs is shown on the last page of the thesis.

Fig. 38 GRAFT REMOVED IMMEDIATELY AFTER TRANSPLANTATION

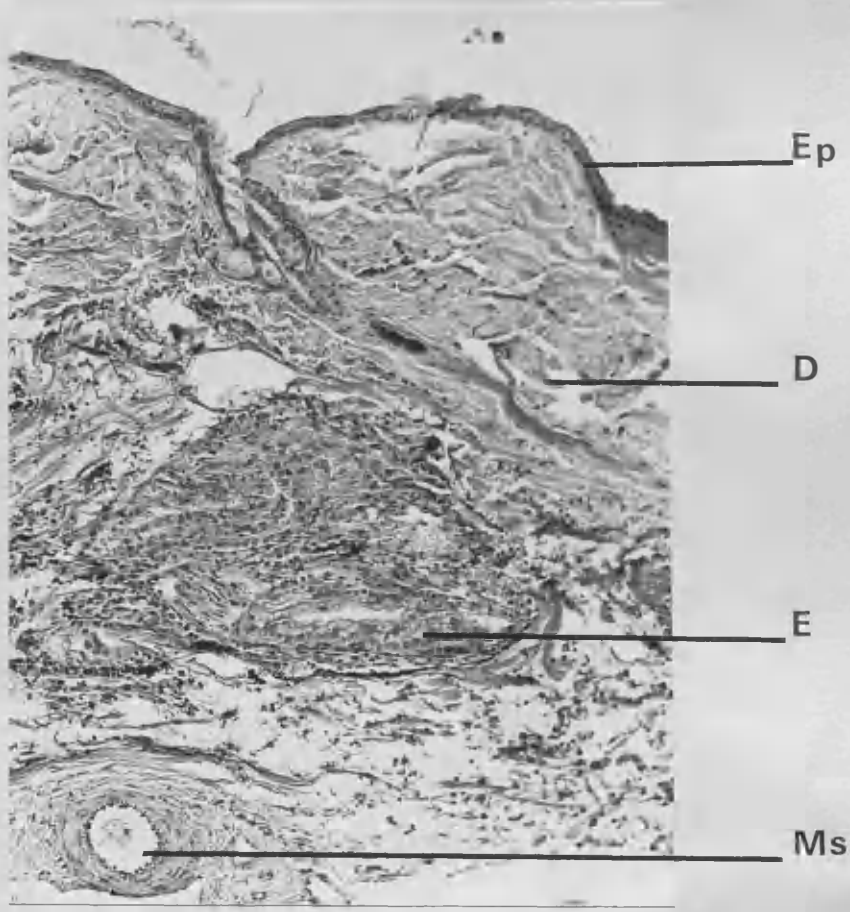
PAS x 100

The graft lies in the deepest part of the dermis. The epidermis lies above the graft. Below the graft there is a medium sized artery lying in loose subcutaneous tissue. The graft is composed of living endodermal cells which are recognised at this stage by their vacuolated apices.

Fig. 39 GRAFT DIR - REMOVED AT 6 DAYS

H & E x 100

The graft lies in the dermis. The central mass of the graft is necrotic. Living endodermal cells form cysts and are present below and to the right of the necrotic mass. The elastic cartilage plate of the ear is shown.


Ep
D
E
Msa

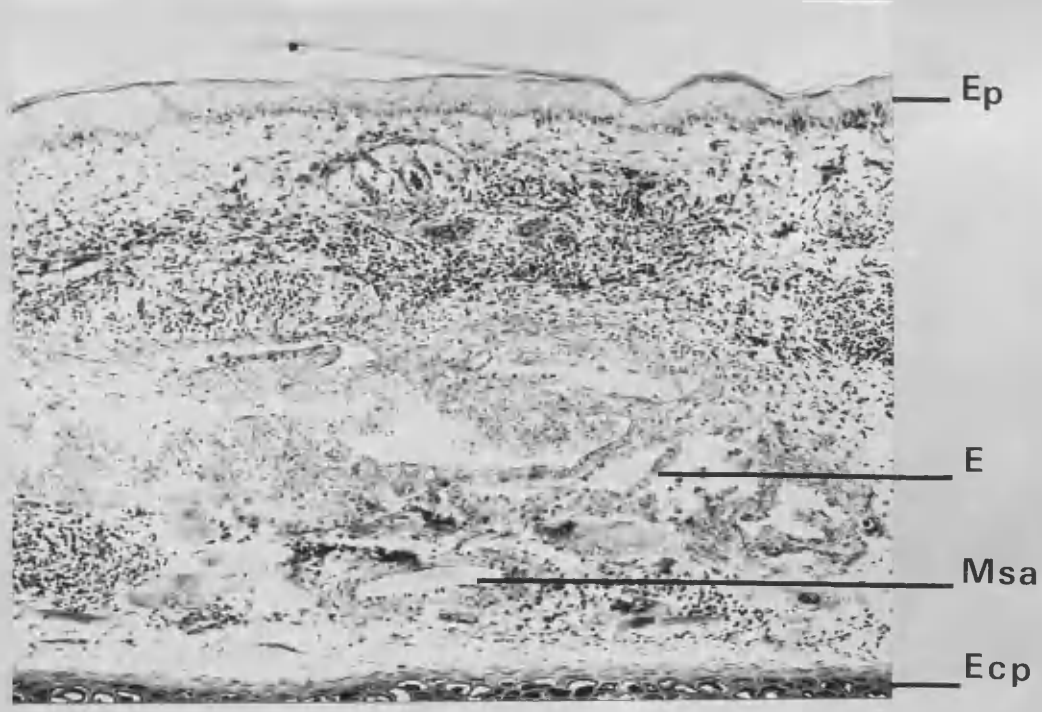

Ep
E
Msa
Ecp

Fig. 40 GRAFT DIR - DETAIL OF FIG. 39

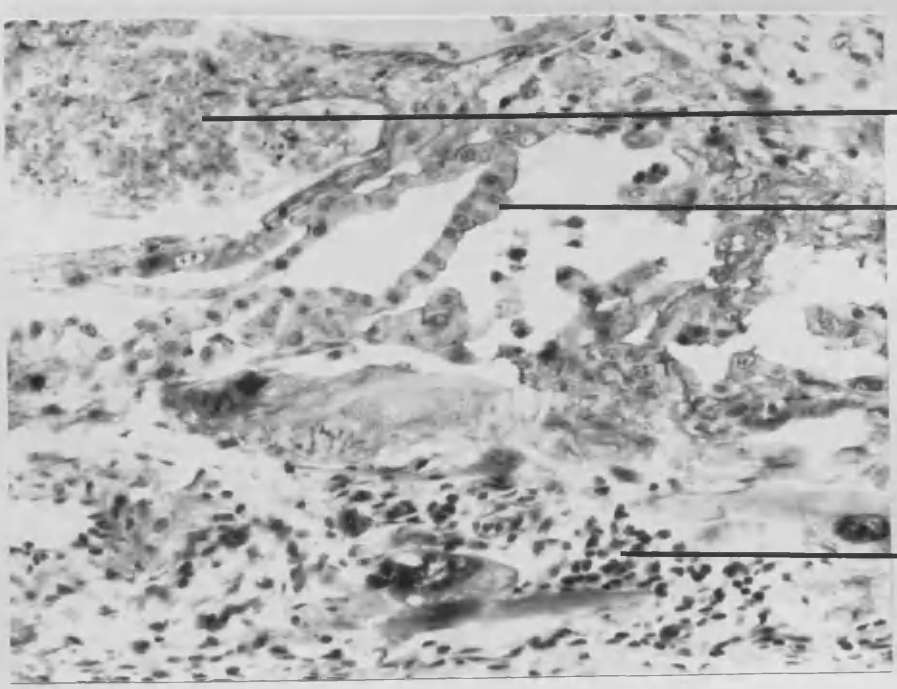
H & E x 250

This photomicrograph shows a collection of living endodermal cells which are now cuboidal in shape. There is a light round cell infiltrate just below the living graft.

Fig. 41 GRAFT DIR - DETAIL OF FIG. 39

H & PAS x 250

This photomicrograph shows a collection of graft giant cells lying in the subcutaneous tissues. Each cell is surrounded by a PAS +ve border. A heavy round cell infiltrate lies below the giant cells and cuffs a small artery in the left lower corner of the photomicrograph.


Ncg
E
Rc

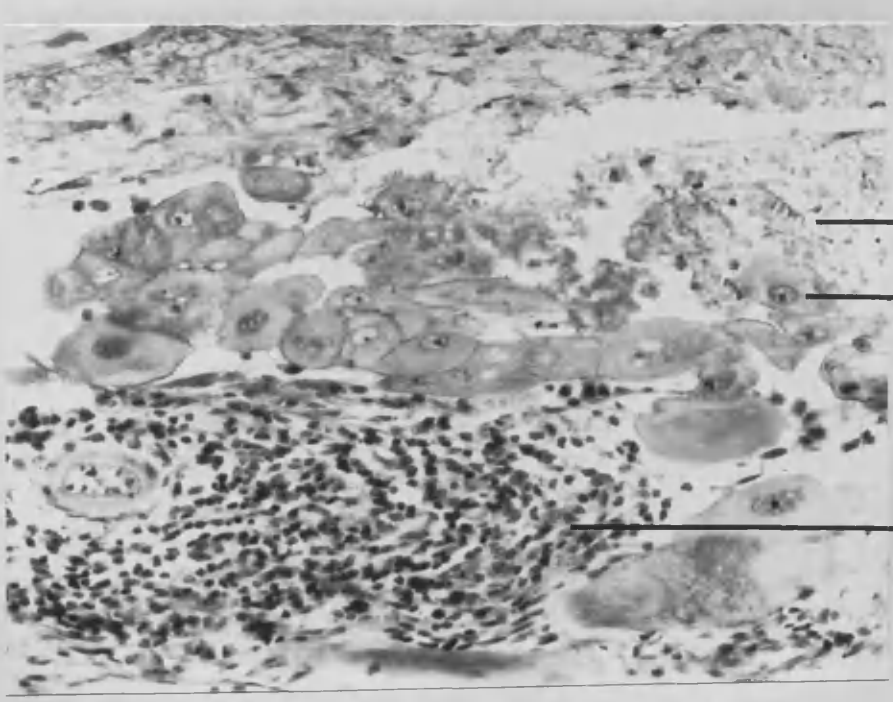

Ncg
Gc
Rc

Fig. 42 GRAFT D2L - REMOVED AT 6 DAYS

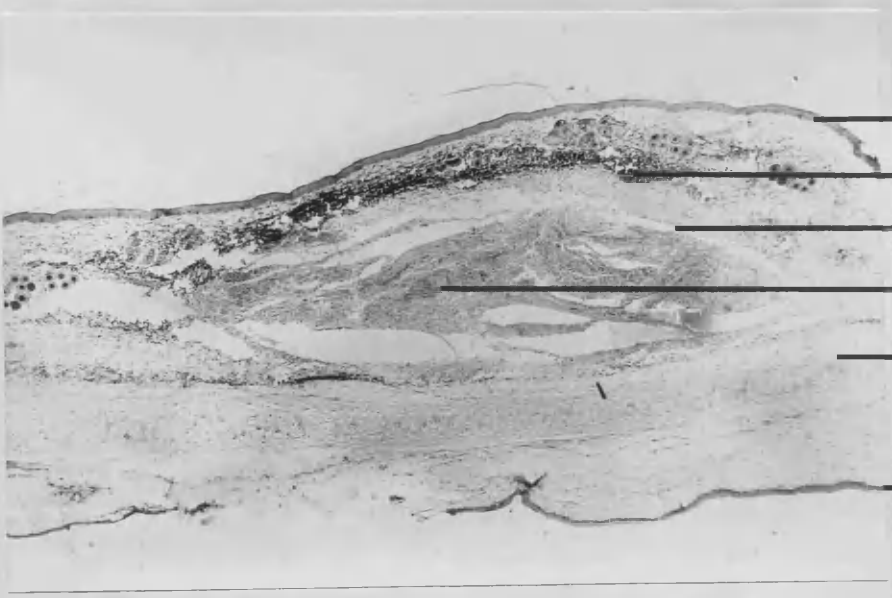
H & E x 50

The whole graft and complete section of the rabbit ear is shown. The graft lies in the dermis on the dorsal aspect of the ear. The elastic cartilage plate separates the ventral and dorsal skin. The central mass of graft is necrotic, but surrounding this there are cysts lined by living endodermal cells. There are no graft giant cells in this particular section.

Fig. 43 GRAFT F4R - REMOVED AT 6 DAYS

H & E x 200

This graft cyst which lies in the superficial dermis is lined on its upper surface by healthy endodermal cells. On the deep surface the cyst is lined by flat pleomorphic variants presumably of endodermal cell origin. A light round cell infiltrate surrounds the whole graft.


Ep
Rc
Gce
Ncg
Ecp
Ep

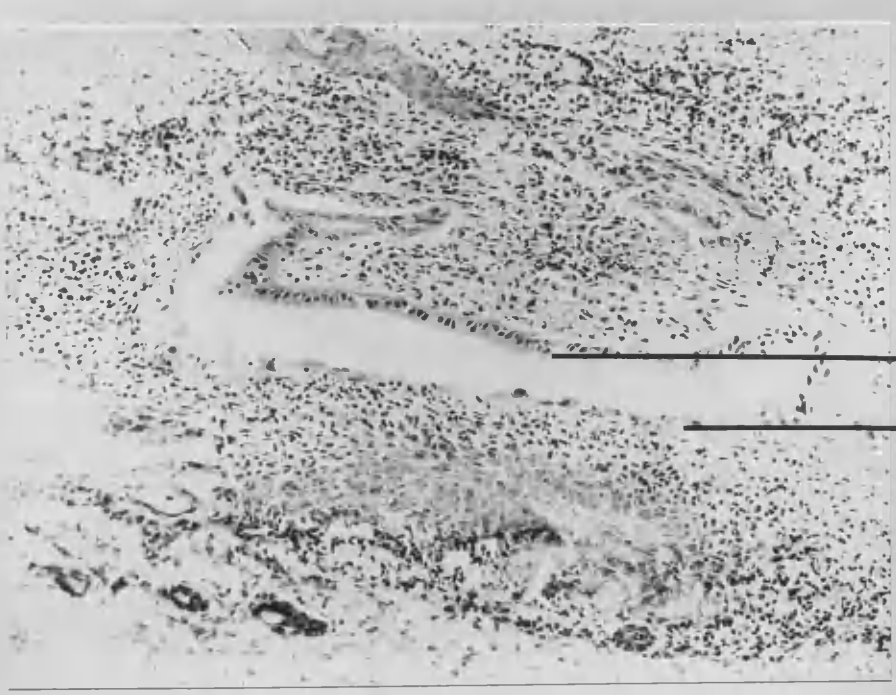

E
Ed

Fig. 44 GRAFT F4R - REMOVED AT 6 DAYS

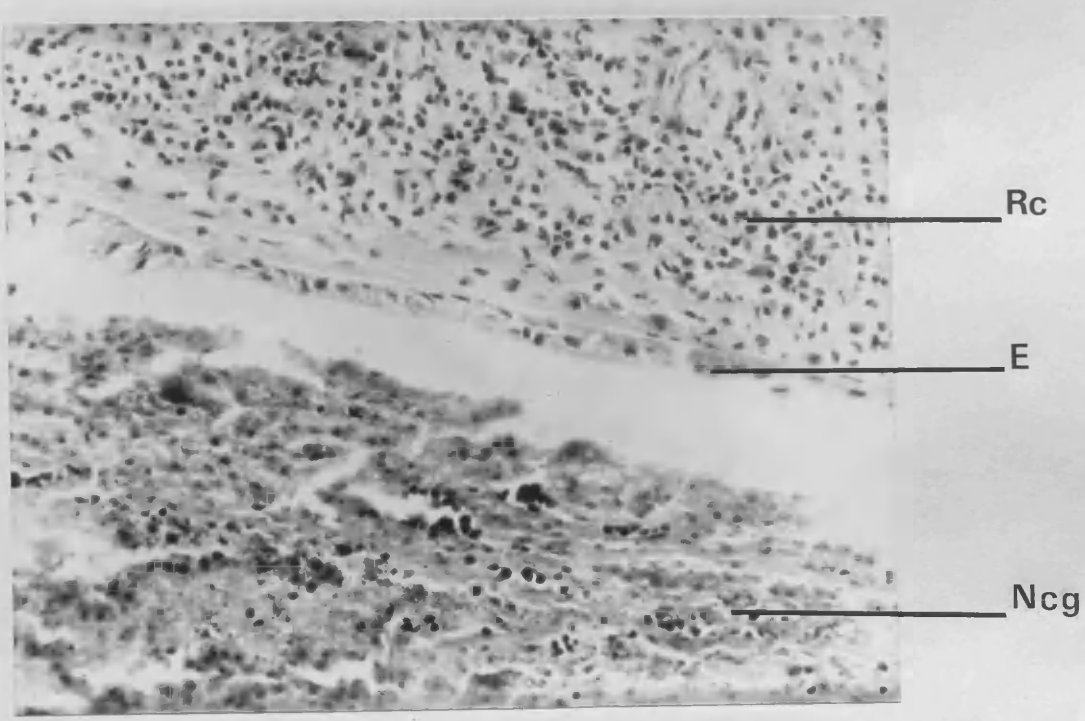
H & E x 250

This photomicrograph shows in the lower half, the main central necrotic mass of graft which stains strongly with eosin. A graft cyst traverses the centre of the photomicrograph. The upper surface of the cyst is lined by living endodermal cells. The dermis in the upper part of the photomicrograph contains a light round cell infiltrate.

Fig. 45 GRAFT F4L - REMOVED AT 6 DAYS

H & E x 180

This photomicrograph shows several cysts lined by living endodermal cells. The collection of darkly staining cells lying just above the graft cyst in the centre of the picture consists mainly of small lymphocytes. A light round cell infiltrate surrounds the graft cysts at the periphery of the central necrotic mass.


Rc
E
Ncg

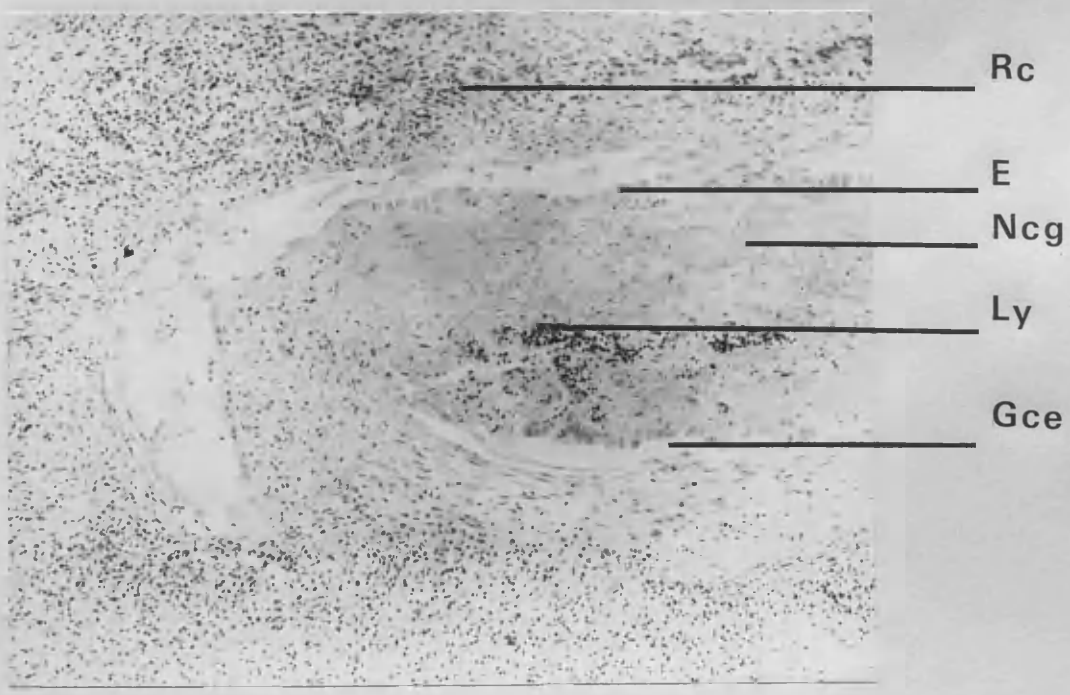

Rc
E
Ncg
Ly
Gce

Fig. 46 GRAFT F4L - DETAIL OF FIG. 45

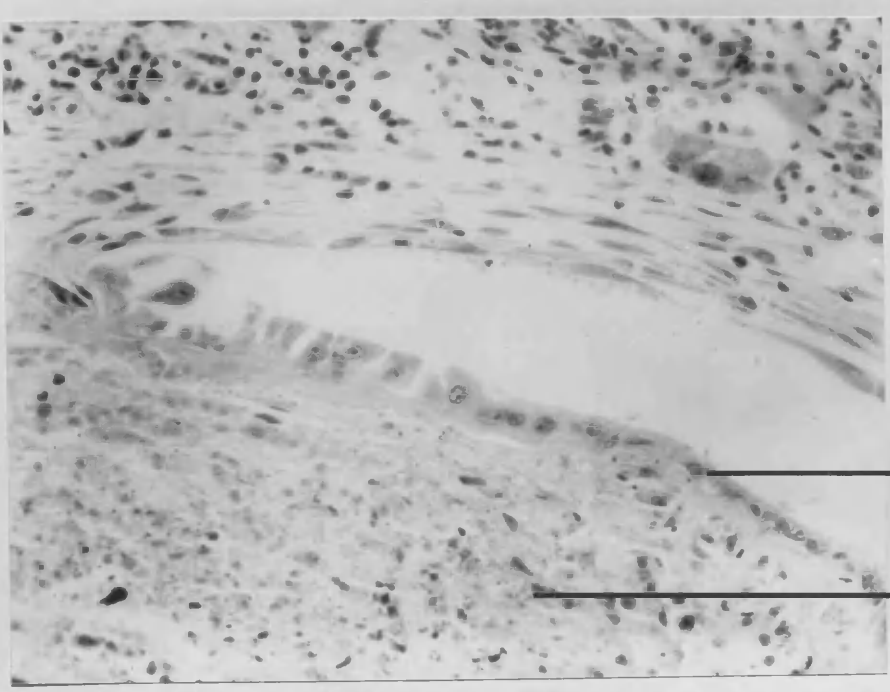
H & E x 250

This is taken from the upper right hand corner of Fig. 45 and shows a cyst lined by living endodermal cells. The lower part of the photomicrograph shows dead graft tissue. The upper part shows the surrounding dermis infiltrated by round cells.

Fig. 47 GRAFT F4L - DETAIL OF FIG. 45

H & E x 400

This photomicrograph shows one type of pleomorphic variant commonly found in the graft cysts. These are endodermal cells which appear unhealthy and are sloughing into the cyst cavity.


E
Ncg

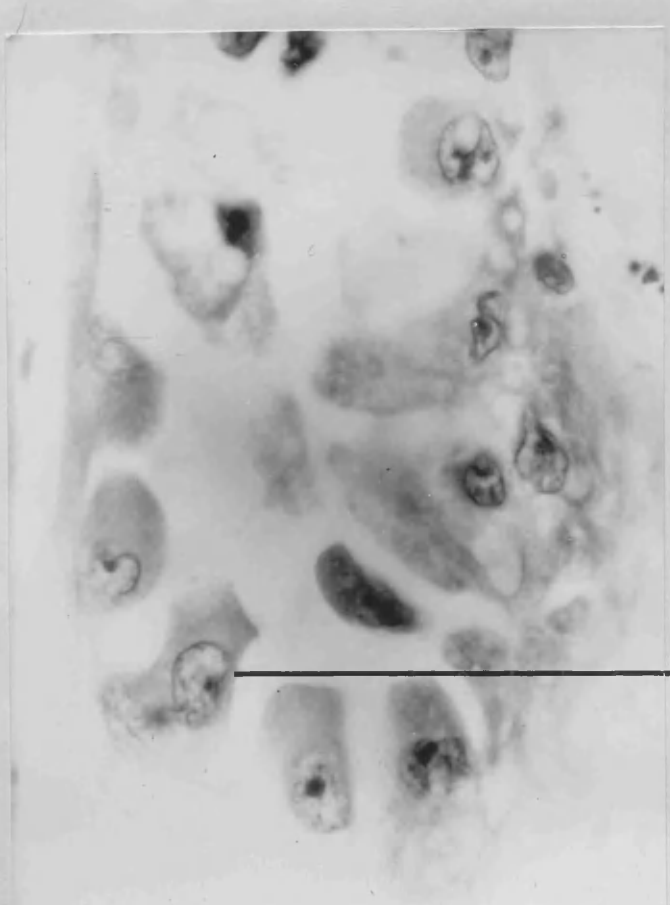

Pv

Fig. 48 GRAFT F5R - REMOVED AT 6 DAYS

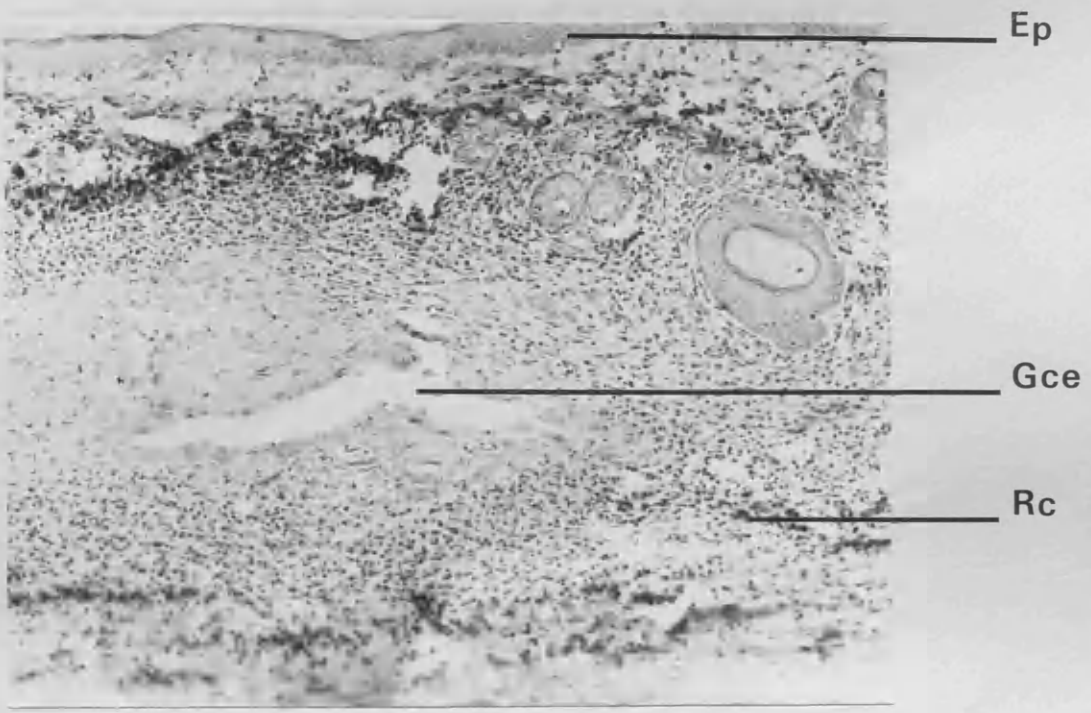
H & E x 100

A small graft cyst lined by living endodermal cells is shown in the centre of the photomicrograph. The cyst is surrounded by a round cell infiltrate.

Fig. 49 GRAFT F6R - REMOVED AT 6 DAYS

H & E x 100

This section shows a small part of the main necrotic mass in the centre of the photomicrograph. Above and below the necrotic mass which contains a vessel packed with infiltrating small lymphocytes, there are graft cysts lined in parts by living endodermal cells.


Ep
Gce
Rc

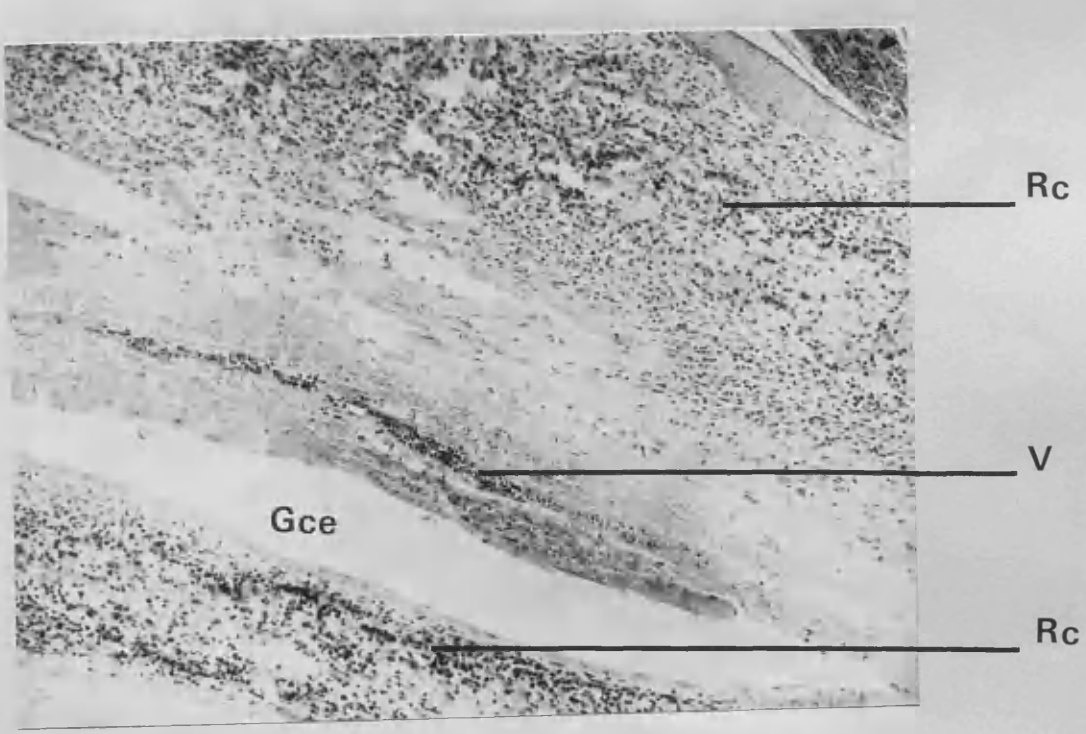

Rc
V
Gce
Rc

Fig. 50 GRAFT F6R - REMOVED AT 6 DAYS

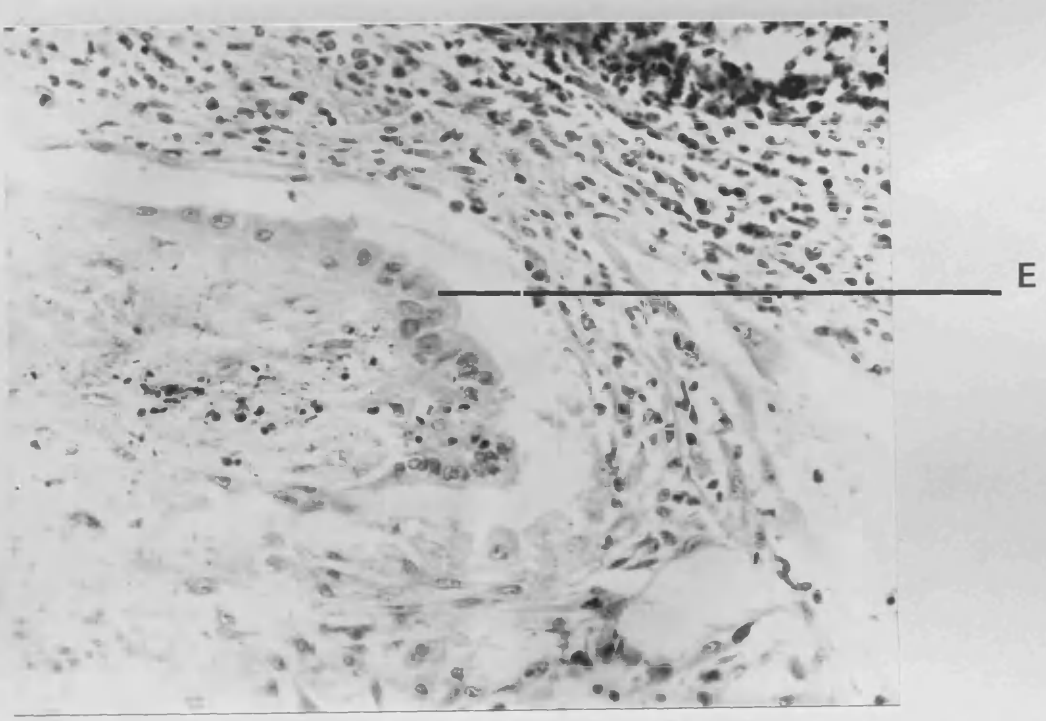
H & E x 250

This is a different section of graft F6R and confirms that the graft contains healthy living endodermal cells.

Fig. 51 GRAFT F6L - REMOVED AT 6 DAYS

H & E x 250

Living endodermal cells are shown whilst in the lower left corner there is necrotic graft infiltrated by a few lymphocytes and macrophages.


E

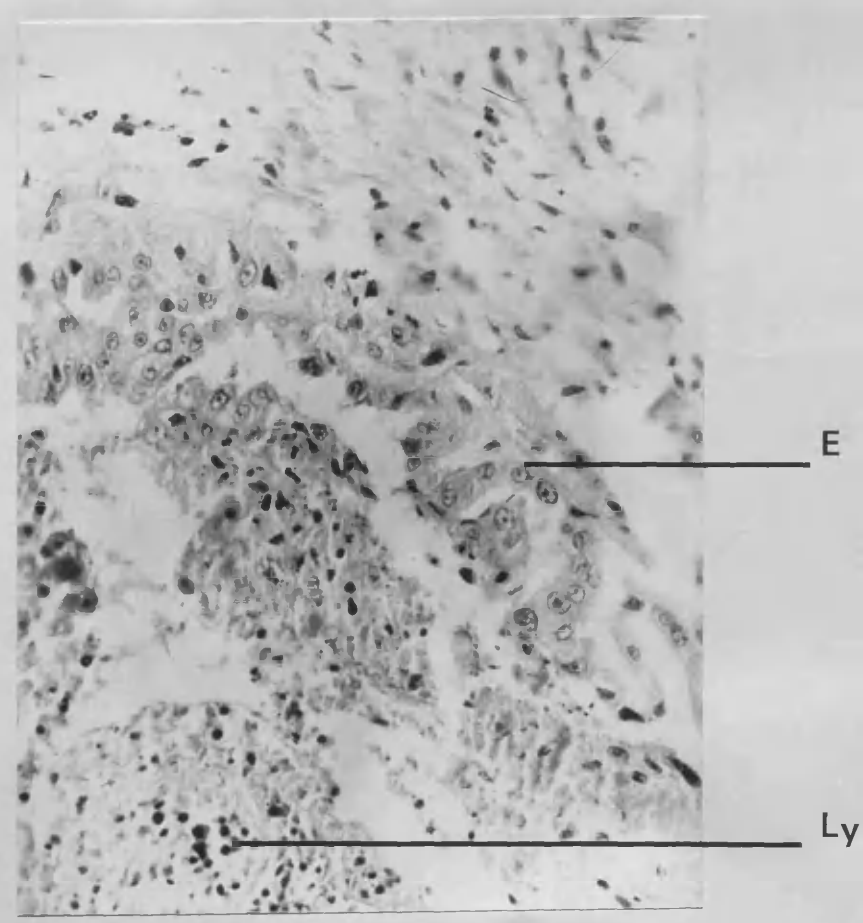

E
Ly

Fig. 52 GRAFT CIR - REMOVED AT 14 DAYS

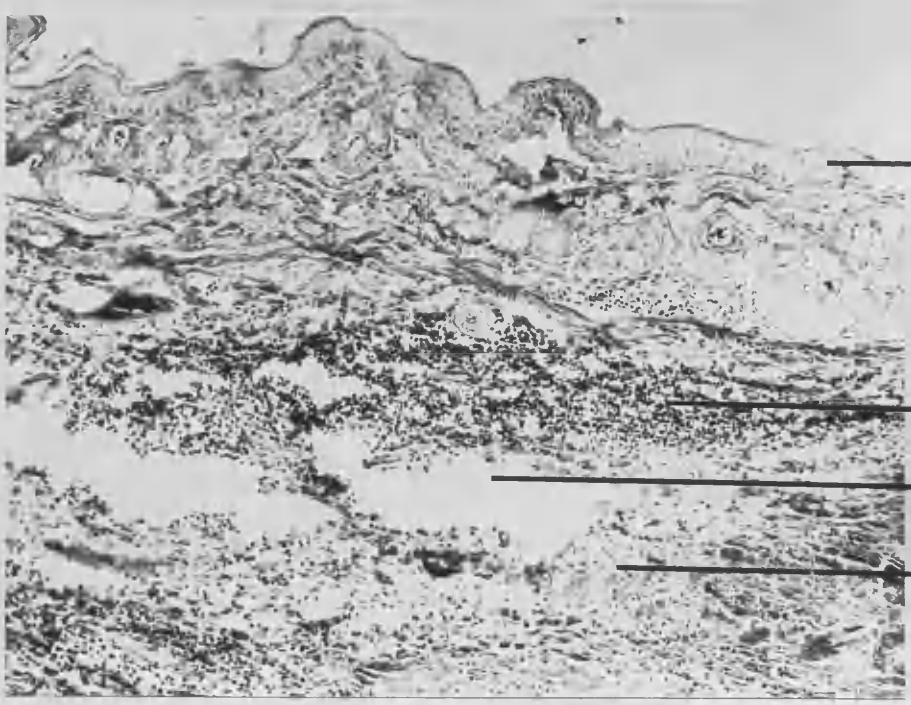
H & E x 100

This photomicrograph shows the typical appearance of the graft at 14 days. There are a number of spaces in the centre of the picture. These are presumably the remains of graft cysts. The whole area is surrounded by a dense round cell infiltrate. No living endodermal cells or pleomorphic variants can be seen.

Fig. 53 GRAFT CIR - REMOVED AT 14 DAYS

H & E x 250

This section is taken from a different part of graft CIR. The basal lamina of the dead visceral yolk sac lies convoluted in an old graft cyst containing cell debris and small lymphocytes.


Ep
Rc
Gcd
Ncg

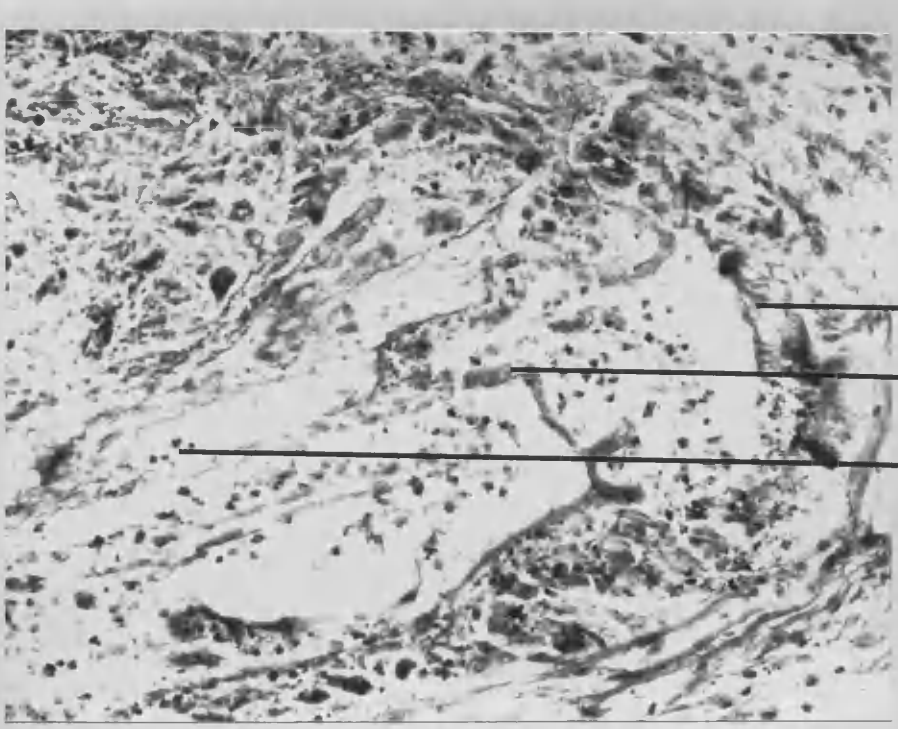

Ncg
Ed
Ly

Fig. 54 GRAFT C1L - REMOVED AT 14 DAYS

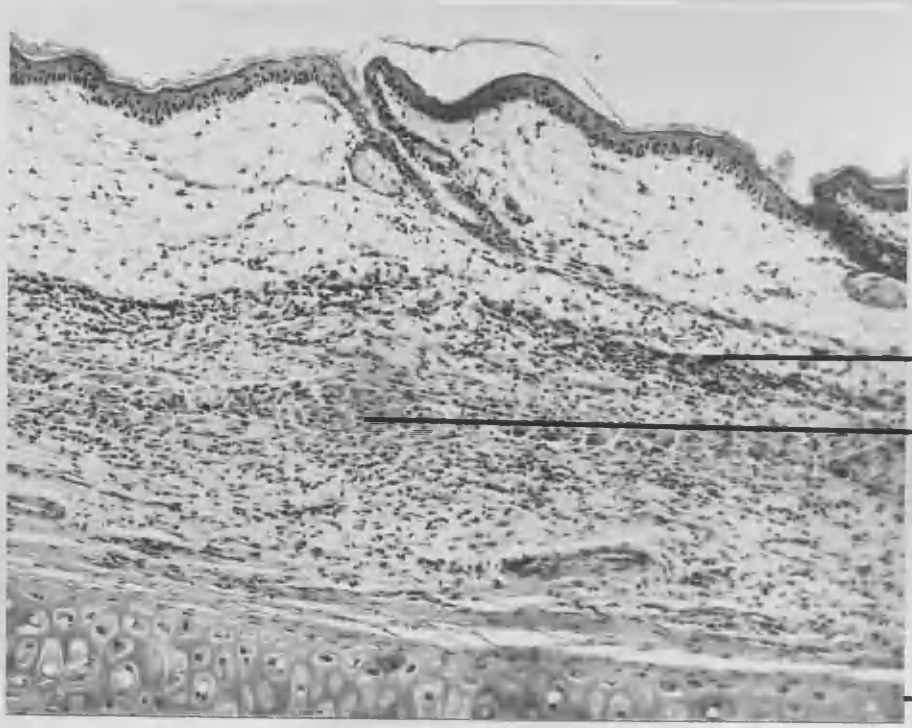
H & E x 100

No living endodermal cells were found in this graft, but as shown in the photomicrograph, only a small amount of graft appears to have been transplanted. There is a round cell infiltrate and the nest of cells in the centre of the photomicrograph is probably dead graft.

Fig. 55 GRAFT C2R - REMOVED AT 14 DAYS

H & E x 63

The central necrotic mass is shown on the left side of the photomicrograph. Old graft cysts surround and lie to the right of the necrotic mass. There is a heavy round cell infiltrate.


Rc
Ncg
Ecp

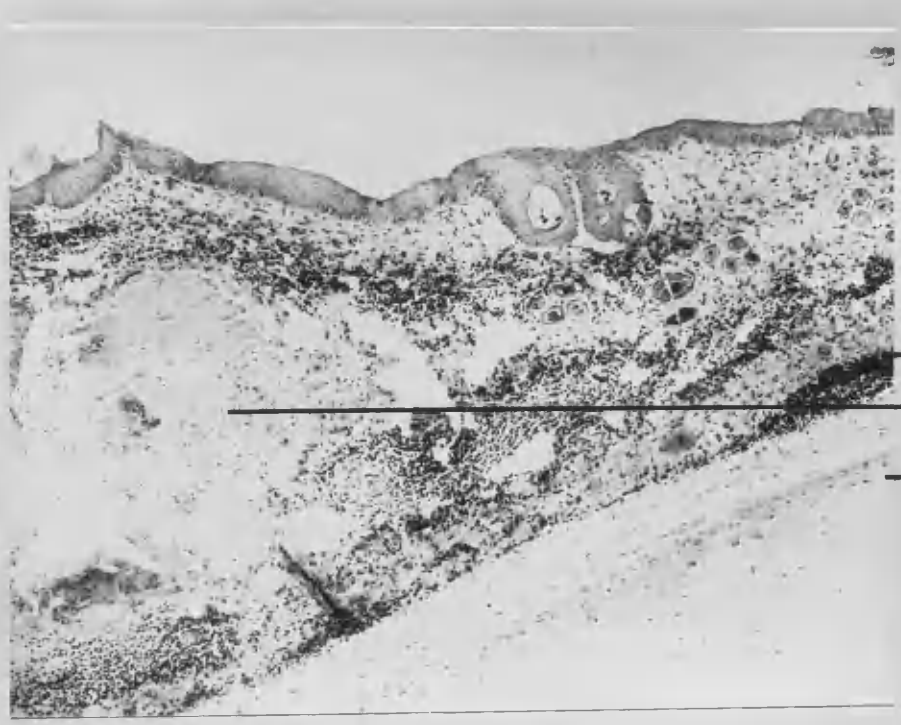

Rc
Ncg
Ecp

Fig. 56 GRAFT C2L - REMOVED AT 14 DAYS

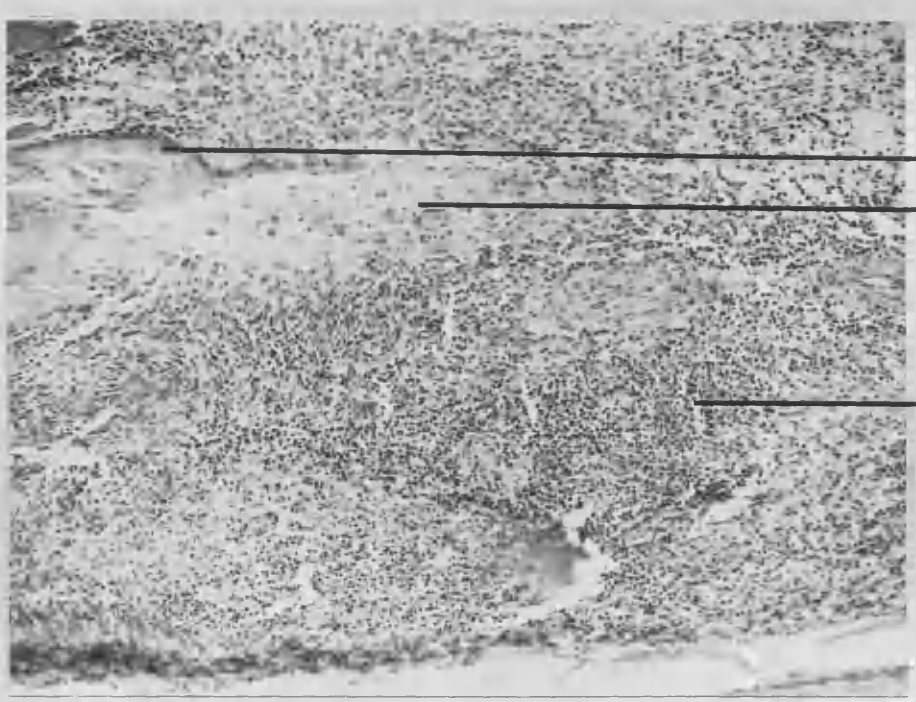
H & E x 100

This photomicrograph shows the open end of a tube of epidermis which has grown from the original puncture site to invest the dead graft. Part of the epidermal tube is shown in the upper right corner. There is a dense round cell infiltrate.

Fig. 57 GRAFT EIR - REMOVED AT 14 DAYS

H & E x 250

This is an example of graft giant cells which lie away from the main mass of necrotic graft. A small collection of lymphocytes is associated with the giant cells.


Ept
Ed
Rc

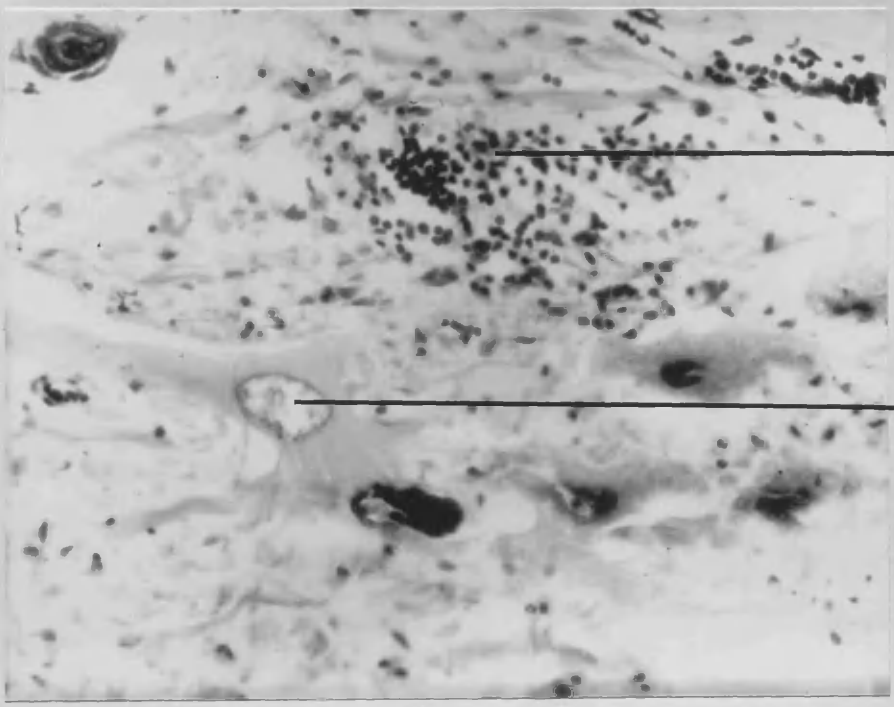

Ly
Gc

Fig. 58 GRAFT E1L - REMOVED AT 14 DAYS

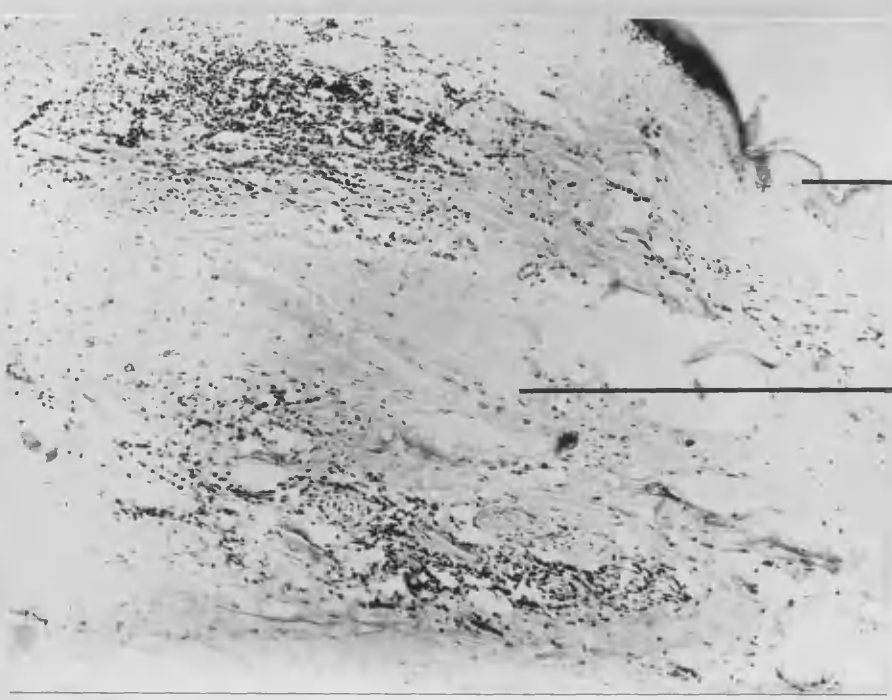
H & E x 100

The dead graft with evidence of old graft cysts is shown in the centre of the photomicrograph. A round cell infiltrate lies associated with a neurovascular bundle below the dead graft. A second collection of round cells lies above the graft.

Fig. 59 GRAFT E2L - REMOVED AT 14 DAYS

H & E x 100

The main necrotic mass of graft is surrounded by a tube of epidermal cells which have migrated from the original puncture site. This is the distal, open end of the tube. Dead endodermal cells are shown in the centre of the photomicrograph.


Ep
Ncg


Ept
Ncg
Ed
Ecp

Fig. 60 GRAFT E3R - REMOVED AT 14 DAYS

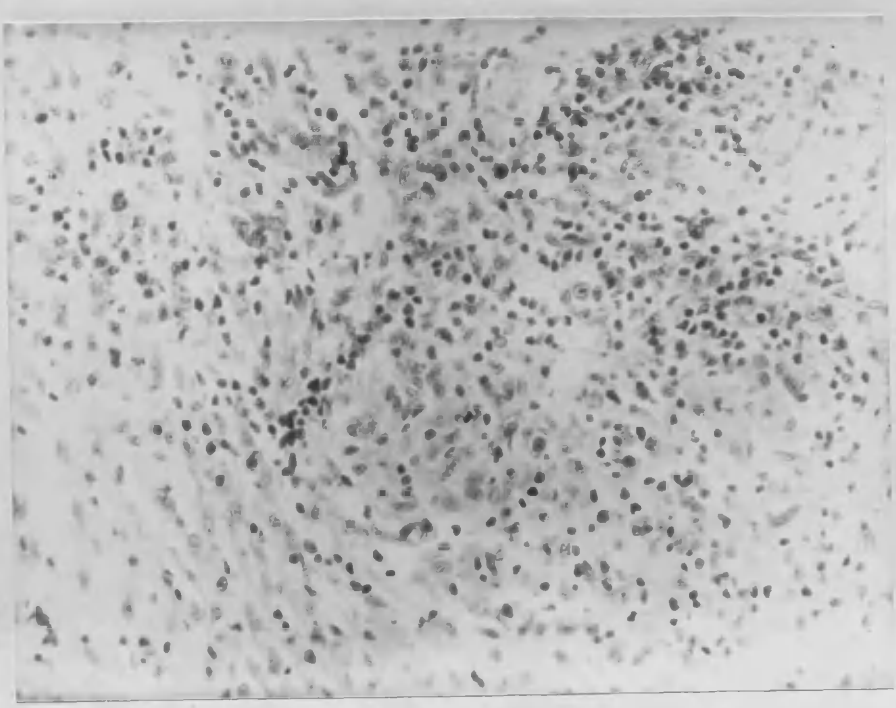
H & E x 250

This photomicrograph shows only a small part of the graft infiltrated by lymphocytes and occasional macrophages.

Fig. 61 GRAFT E3L - REMOVED AT 14 DAYS

H & E x 63

The whole graft is shown surrounded by a dense round cell infiltrate. The lower infiltrate originates from vessels in the subcutaneous tissues. The upper infiltrate originates from vessels lying just below the epidermis. The centre of the necrotic mass contains a light round cell infiltrate. At the periphery of the mass there appear to be old graft cysts. A number of graft giant cells are present in the subcutaneous tissues. There are no living endodermal cells.



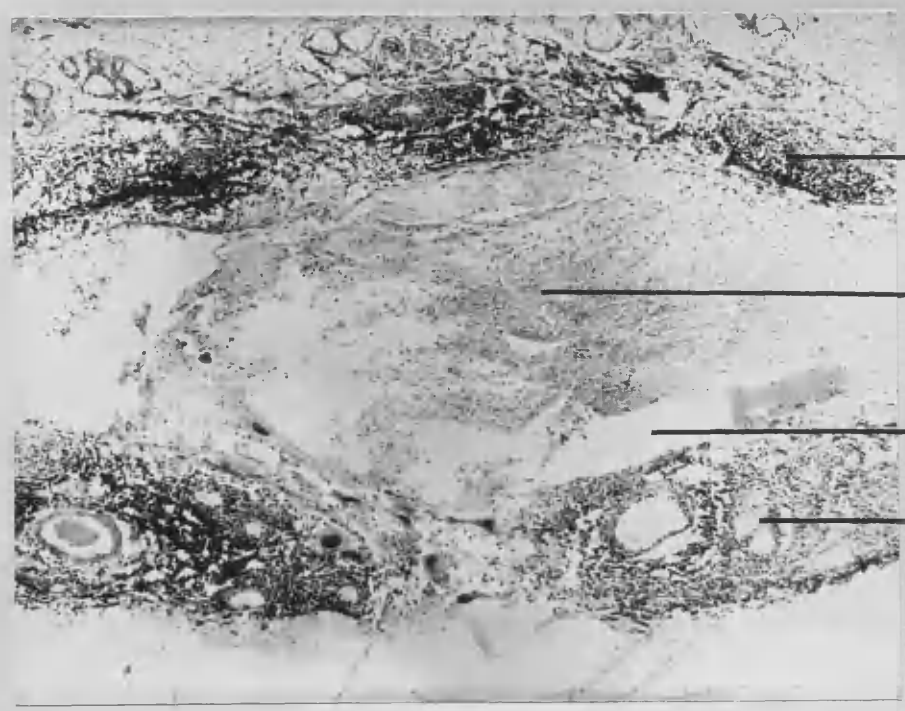

Rc
Ncg
Gcd
Gc

Fig. 62 GRAFT E3L - DETAIL OF FIG. 61

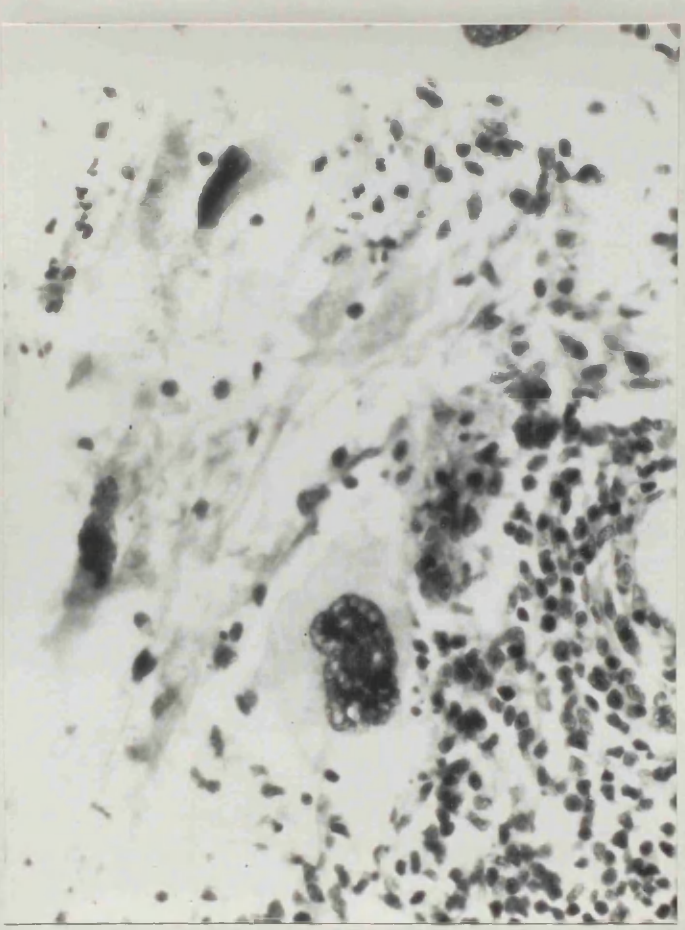
H & E x 400

This shows the round cell infiltrate surrounding a graft giant cell. The round cell infiltrate consists of lymphocytes, macrophages, plasma cells and cells of indeterminate origin.

Fig. 63 GRAFT E3L - DETAIL OF FIG. 61

H & E x 400

This shows the staining properties of the graft giant cells. The cytoplasm appears fibrillary and stains lightly with haematoxylin. An hyaline membrane surrounds the cell. The round cell infiltrate is shown.



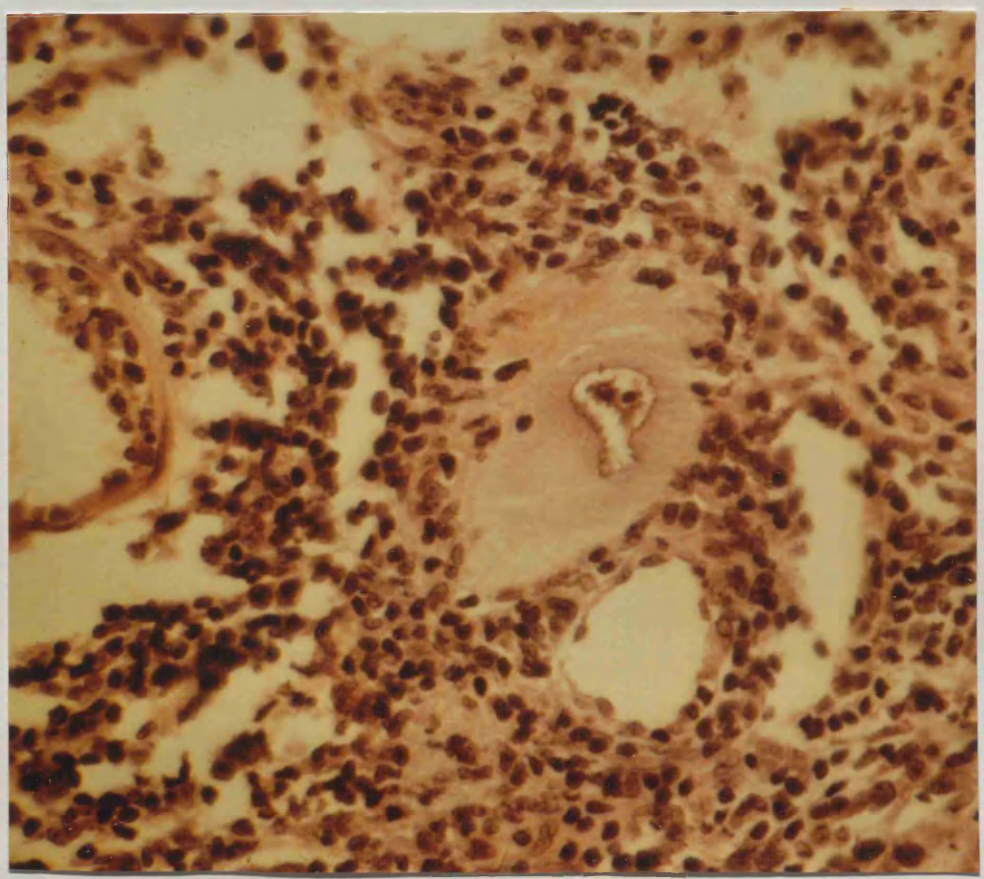


Fig. 64 GRAFT FIR - REMOVED AT 14 DAYS

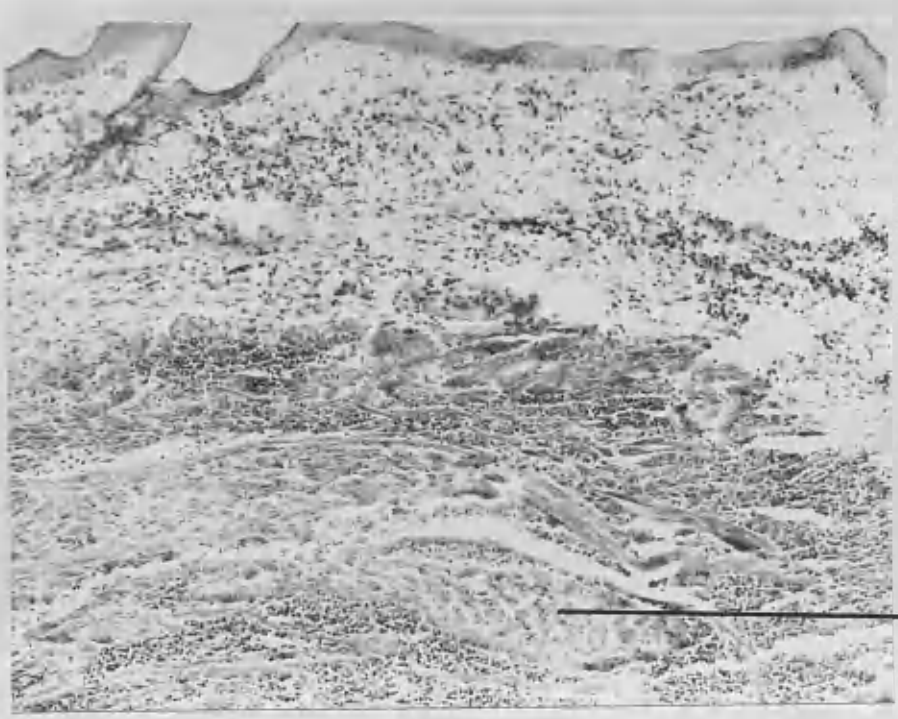
H & E x 100

Dead yolk sac is shown in the lower part of the photomicrograph. Some of the infiltrating cells are polymorphs, but most are round cells.

Fig. 65 GRAFT FIL - REMOVED AT 14 DAYS

H & E x 100

This photomicrograph shows large numbers of dead endodermal cells lying on the convoluted basal lamina of the dead visceral yolk sac.


Ed

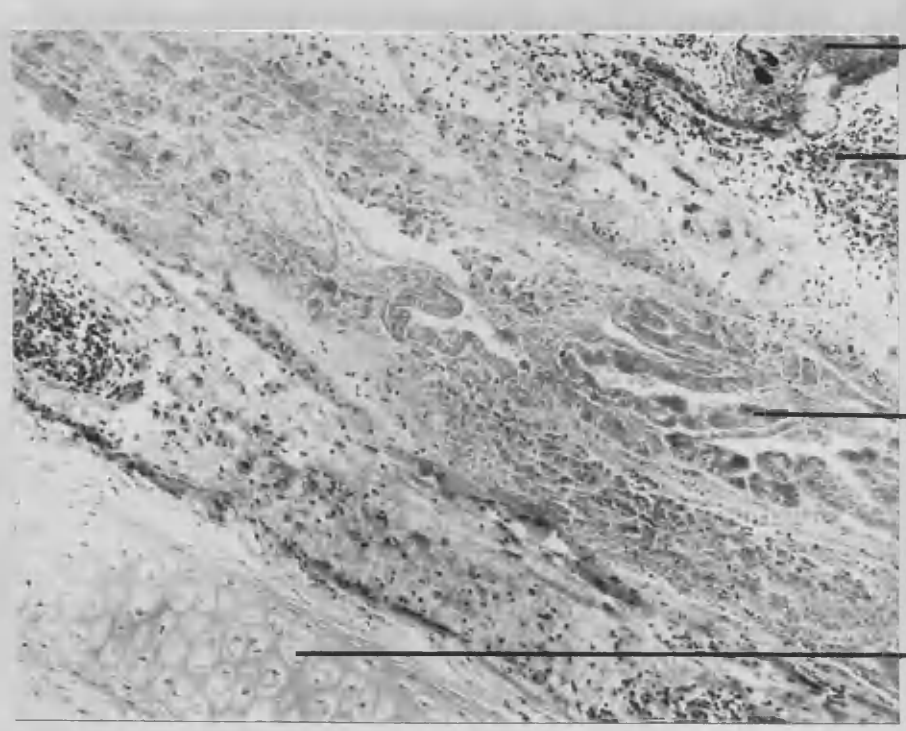

Ep
Rc
Ed
Ecp

Fig. 66 GRAFT F2R - REMOVED AT 14 DAYS

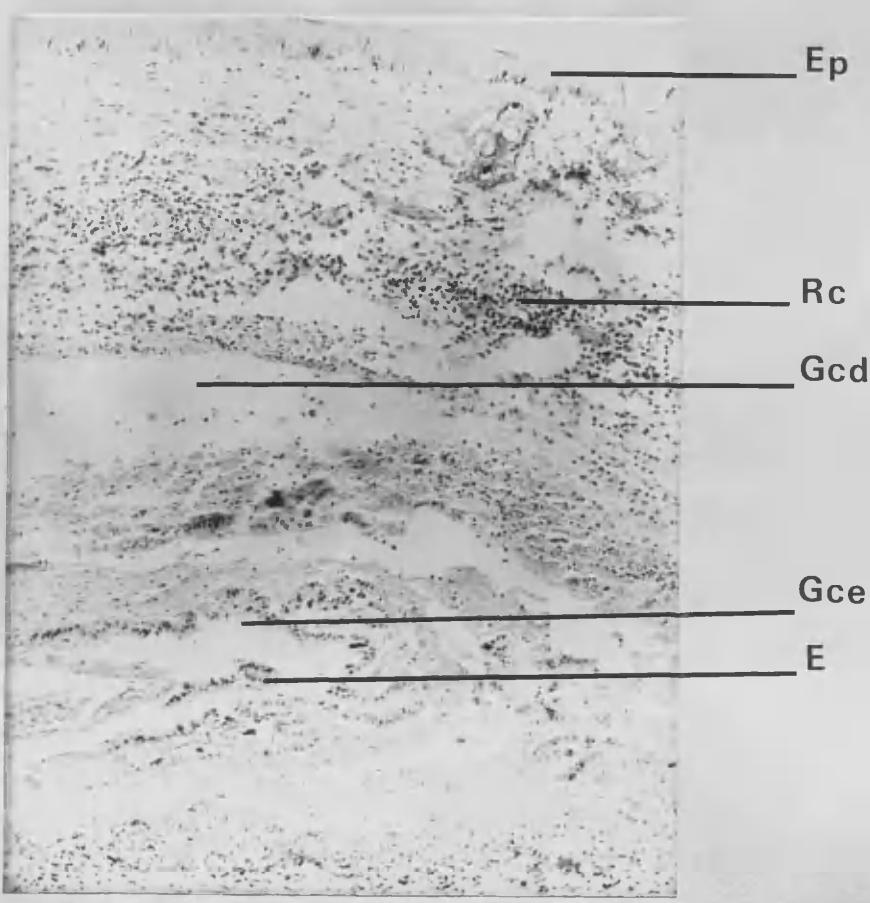
H & E x 100

This photomicrograph shows evidence of old cysts devoid of living endodermal cells, but living endoderm does line a graft cyst. This is shown in the lower half of the photomicrograph.

Fig. 67 GRAFT F2R - DETAIL OF FIG. 68

H & E x 400

This shows the endodermal cells referred to above. The cells appear to be healthy although some are sloughing into the cyst cavity.


Ep
Rc
Gcd
Gce
E

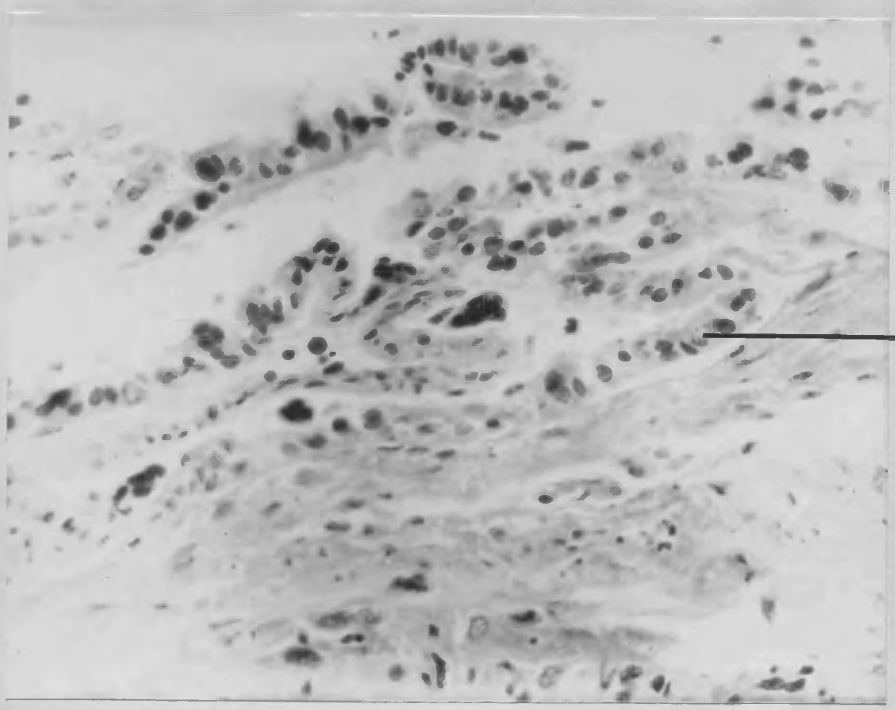

E

Fig. 68 GRAFT F2L - REMOVED AT 14 DAYS

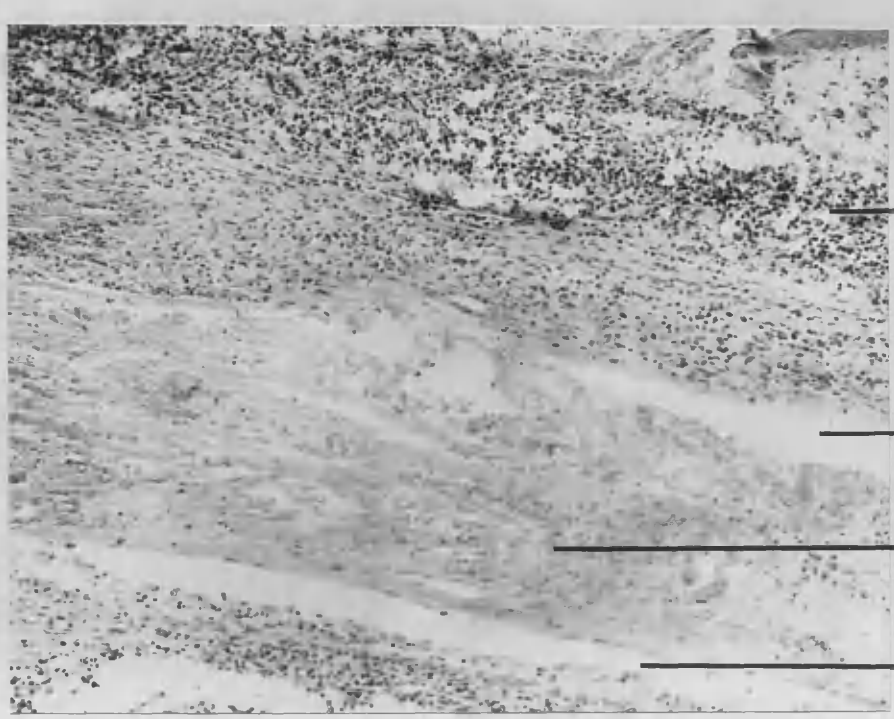
H & E x 100

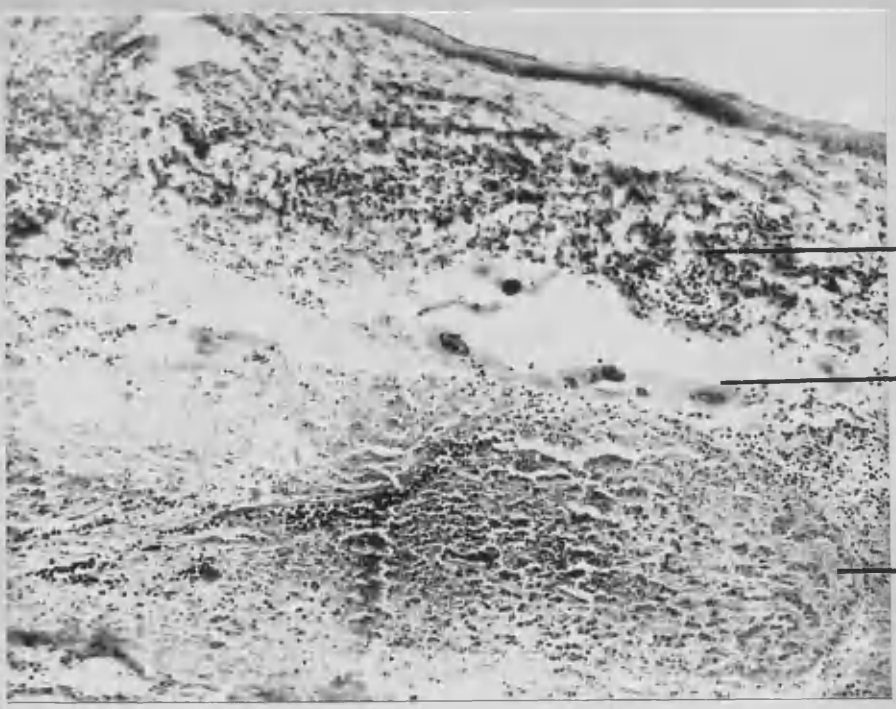
The graft is necrotic. No living endodermal cells are seen. The graft is surrounded by a heavy round cell infiltrate.

Fig. 69 GRAFT F3L - REMOVED AT 14 DAYS

H & E x 100

The graft is necrotic and infiltrated by round cells. There is a collection of dead endodermal cells in the right lower corner of the photomicrograph.


Rc
Gcd
Ncg
Gcd


Rc
Gc
Ed

Key to the photomicrographs of the grafting study.

D - Dermis of rabbit ear

E - Living endodermal cells

Ed - Dead endodermal cells

Ecp - Elastic cartilage plate of rabbit ear

Ep - Epidermis

Ept - Part of an epidermal tube surrounding the graft

Gc - Graft giant cell

Gce - Graft cyst containing living endodermal cells

Gcd - Graft cyst devoid of living endodermal cells

Ly - Lymphocytes

Msa - Medium sized artery

Ncg - Necrotic graft

Pv - Pleomorphic variants of endodermal cells

Rc - Round cell infiltrate

S - Subcutaneous tissues of rabbit ear

V - Vessel packed with small lymphocytes