HISTOLOGICAL LOCALIZATION OF THE ANTIPATERNAL TYPE H-2 ANTIBODY BINDING IN VIVO IN THE MURINE PLACENTA

by

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ANTIPATERNAL H-2 ANTIBODY BINDING IN VIVO IN THE MURINE PLACENTA.

Abstract

The presence of paternal class 1 MHC antigens (Ag) in situ on various cells of the 15 day murine placenta and the precise location of the antigenic sites were examined by an evaluation of their temporal (2 minutes-2 hours) labeling patterns at the light and electron microscope levels after a single intravenous injection of I^{125}-labeled monoclonal anti-H-$2K^K$ antibody (Ab) into C57BL/6 ♀ mice impregnated with CBA or C57BL/6 males. The results revealed the presence of H-2 Ag on the plasma membrane of all the three layers of (trichorial) labyrinthine trophoblasts, of spongiotrophoblasts, fetal stromal cell clusters, macrophages and endothelium of fetal capillaries, but not on trophoblast giant cells. Some of the trophoblast antigenic sites were located on microvilli. Labeling of stromal cells, endothelium and macrophages was partly explained by binding of Ag-Ab complexes via their Fc receptors. Labeled Ab or Ag-Ab complexes were internalized by most cells, but some degradation was noted in macrophages and some trophoblast cells. In vitro binding of radiolabeled anti-H-2 or unrelated (anti-HLA-DR) Ab to dispersed placentae in the presence or absence of excess mouse IgG indicated an equal incidence of Fc receptors (FcR) in homozygous and heterozygous placentae, but the affinity of Ab binding via FcR was found to be weaker than via H-2 sites both at 4°C and 37°C. Both placental types acted as potent barriers against the passage of Ab molecules to the fetus, but such a barrier function was not selective for the antifetal type Ab. The barrier function was most likely exerted by fetal stromal cells, endothelial cells and macrophages.

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RESUME

La présence d'antigènes (ag) paternels de class 1 MHC in situ sur diverses cellules placentaires de la souris à 15 jours de gestation et la localisation exacte des sites antigeniques ont été investiguées par une evaluation des modes d'impregnation a diverses périodes (2 min - 2 heures au microscope optique et electronique), après injection intraveineuse unique d'anticorps (ac) monoclonaux anti-H-2K^{k} marqués à I^{125} dans les souris femelles C57BL/6 fecondées par des mâles CBA ou C57BL/6. Les résultats montrent la présence d'antigènes H-2 sur les plasmalemmes des trois couches de trophoblastes labyrinthiques (trichoriale), de spongiotrophoblastes, des groupes de cellules stromales foetales, de macrophages et de l'endothelium des capillaires foetaux alors que les cellules géantes trophoblastiques n'en montrent pas. De plus, quelques sites antigeniques des trophoblastes situés sur des microvillosités ont aussi été marqués. Le marquage des cellules stromales de l'endothelium et des macrophages peut-être en partie expliqué par des liaisons de complexes ag-ac par l'intermédiaire de leurs récepteurs Fc. Les ac et les complexes ag-ac marqués étaient incorporés par la plupart des cellules, quoiqu' une certaine dégradation était observée dans les macrophages et les cellules trophoblastiques. Les deux types de placenta homozygote et hétérozygote offraient une forte résistance au passage d'anticorps vers le foetus, cependant cette barrière n'était pas selective pour l'ac de type antifoetale. Cette fonction de barrière était fort probablement le résultat d'une résistance offerte par les cellules stromales, endotheliales et les macrophages.

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To my family.

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PART 1 INTRODUCTION

1.1 Nature's Most Commonplace Allograft

The implantation of an embryo in the uterine endometrium and its development and survival as a feto-placental unit for the duration of gestation is an enigma that has for decades puzzled transplantation and reproductive biologists. The conceptus resulting from mating in a genetically outbred mammalian population is an allograft with respect to the mother because of the inheritance of histocompatibility antigens of both parental allotypes by the offspring in a codominant manner. Nevertheless, the intrauterine fetal allograft does not evoke a rejection response in the mother, although, during pregnancy, she is perfectly capable of rejecting, at other sites, grafts genetically similar to her fetus. This paradox has been the focus of investigation for many years leading to several hypotheses, some of which will be discussed later on.

An understanding of the mechanisms of success of the most commonplace allograft in nature has several practical implications:

1. application in improving clinical allotransplantation
2. improvement in fetal and neonatal survival and
3. possible elucidation of the immunological escape and subsequent growth of neoplasms, which may use several mechanisms.

1.2 Mechanisms of Allograft Rejection

In general when a tissue is transplanted onto a host which is genetically nonidentical to the donor, the tissue is rejected. Rejection of a foreign tissue graft by the host is an immunological event which is mediated by thymus-derived T cells. Certain clones of T cells will recognize particular surface antigens on the grafted tissue

as non-self and give rise to effector cells mediating the graft rejection. These surface antigens are glycoproteins embedded in the cell membrane and are encoded by the Major Histocompatibility Complex (MHC) loci. These molecules are known as major histocompatibility antigens: H-2 in mice and HLA in humans (Figure 1). There are two classes of MHC antigens 1) Class 1 antigens i.e. H-2 K D and L in mice and HLA-A, B and C in humans 2) Class 2 antigens i.e. Ia in mice and HLA D/DR in humans. Class 1 antigens are the targets for cytotoxic T cell killing, are found on cells in almost every tissue of the body and are primary requirements for an allograft response. Class 2 antigens are found on certain cells eg. B cells, macrophages and some other accessory cells of the immune system and are only needed to produce a good rejection response against a primary allograft (Bach 1980)

Precursors of cytotoxic T cells (P-Tc) bearing specific receptors for class 1 antigens on the graft, when confronted with the foreign antigens undergo clonal proliferation and amplification with the help of T helper cells. At the same time, precursors of T helper cells (P-Th) which bear receptors for the specific class 2 antigens on the graft, will also undergo clonal proliferation and amplification when they encounter the appropriate antigen, giving rise to functional T helper cells which produce interleukin 2(IL-2) (Figure 2). It is believed that after being triggered by the class 1 molecules, P-Tc express receptors for IL-2, binding of which allows a further amplification and maturation into cytotoxic T cells (Tc). Tc, upon recognition of cells bearing the stimulatory target antigens, will

FIGURE 1

Loci of the Major Histocompatibility Complex (MHC) of the mouse located on chromosome 17 (courtesy of Saswati Chatterjee Hasrouni, reproduced from Ph.D. thesis, The Immunobiology of Feto-Maternal Relationships, 1981). The location of I-J remains questionable. Recent studies (Hayes et al., 1984; see also review by Sachs et al., 1984) may suggest that two complementing genes, one linked with MHC and the other on chromosome 4 may control I-J antigen expression on cells.

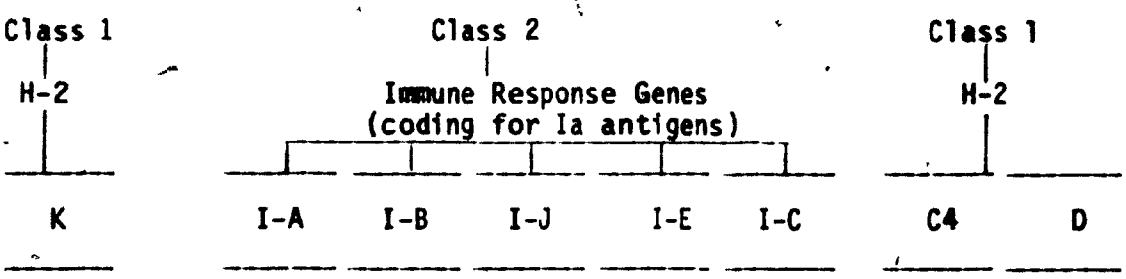
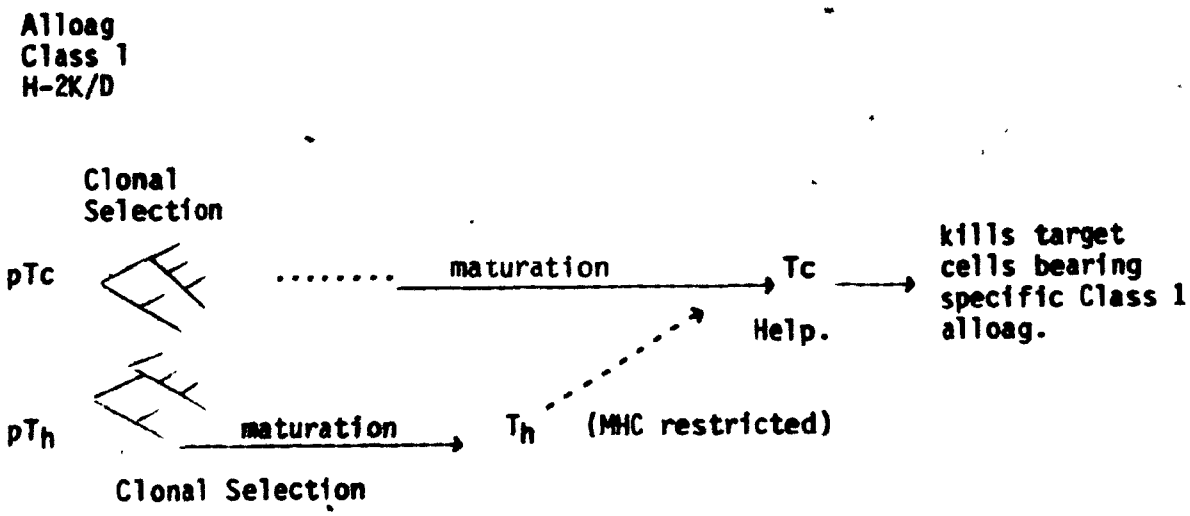

Class 1
H-2
K
Class 2
Immune Response Genes
(coding for Ia antigens)
I-A
I-B
I-J
I-E
I-C
Class 1
H-2
C4
D

Figure 2. MAJOR EVENTS LEADING TO THE ACTIVATION OF T LYMPHOCYTES DURING ALLOSENSITIZATION



Tc cytotoxic T lymphocytes
pTc precursors of cytotoxic T lymphocytes
T_h helper T lymphocytes
pT_h precursors of helper T cells

kill and lyse cells of the graft (Alter and Bach, 1979). The series of main events leading to an allograft rejection is shown in Figure 2.

Other mechanisms which may also play a role in the destruction of an allograft are the production of cytotoxic antibodies, and subsequent complement-mediated lysis and/or antibody dependent cellular cytotoxicity. Furthermore, T cells involved in the delayed type hypersensitivity response, when stimulated by recognition of MHC antigens on the graft, may release factors which act as signals for the recruitment of cytotoxic macrophages (Loveland et al. 1981).

1.3 The Placenta

1.3.1 Function and Anatomy

The placenta is a transient organ that allows an exchange of physiologically important molecules between the mother and developing embryo. It is entirely fetal in origin and its engraftment on the maternal reproductive tract separates the fetus from the maternal cells. Maternal and fetal circulations, both of which pass through the placenta, remain completely separated by a tissue barrier known as the "placental barrier" which acts as the feto-maternal interface, so that all exchanges of material must take place across this interface (Boyd and Hamilton, 1970). This interface also acts as a protective filter preventing harmful substances from reaching the fetus.

1.3.2 Types of Placenta

Two major types of placenta exist; the yolk sac (or chorio-vitalline) and the chorionic (or chorio-allantoic) placenta (Arey, 1965). In the latter placenta, the placental trophoblast cells which

are of fetal origin constitute the immediate cellular boundary between embryonic and maternal tissues throughout most of pregnancy.

Chorionic placentae have been further classified on the basis of the structure of the various tissue layers in the placental barrier (Arey, 1965). When the chorion is apposed to an intact endometrium, the relationship is called epitheliochorial (e.g., pig, horse). Ruminant placentae earlier described "syndesmochorial" (in which trophoblast cells are believed to breach the uterine epithelium and embed in the endometrial stroma) are now found to be epitheliochorial. In the endotheliochorial placenta (carnivores), the trophoblast is in close association with the maternal endothelium. In the hemochorial placenta (bats, higher primates, some insectivores, and rodents), the maternal blood comes into direct contact with the trophoblast tissue. In hemo-endothelial placentae (higher rodents) the trophoblast layer may be eroded, exposing fetal capillaries to the maternal blood. Since the present study has been performed on the mouse placenta, a brief description will be provided on the cell lineage studies of the placenta in this species.

1.3.3. Cell Lineage Studies of the Murine Placenta

In the transitional period between 8 cell stage and morula (32 cell) stage of the embryo, the development of progenitors of two distinct cell lineages first becomes apparent: an outer shell of cells (trophectoderm) surrounds an inner core of cells, the embryonic cells or progenitors of the inner cell mass (ICM). At 3 1/2 days post coitum, cells of the trophectoderm are polygonal cells held together by tight junctions. This gives shape to a hollow spherical blastocyst

containing a fluid filled cavity known as the blastocele. The blastocyst lies free in the uterine lumen at this stage, and is separated from maternal tissues by a mucopolysaccharide coat, the Zona pellucida. By the 4th day, as soon as the blastocele has developed, two populations of trophectoderm may be distinquished: the polar trophectoderm overlying the ICM, and the mural trophectoderm in association with the blastocele. Initially, the mural and polar trophectoderm cells will have similar properties (Gardner et al., 1973), though once the Zona pellucida is lost their individual fate is different. On day 4, the blastocyst sheds its zona pellucida and later on the trophectoderm attaches on the antimesometrial wall of the uterus. After implantation, the mural trophectoderm cells stop dividing and form the primary mononuclear trophoblast giant cells. Biochemical evidence indicates that during giant cell transformation repeated endoreduplication of the entire genome occurs (Sherman et al., 1972).

The polar trophectoderm, however continues to divide, eventually forming the ectoplacental cone (EPC). Gardner et. al. (1973) proposed that the diploid EPC trophoblast cells transform into the secondary giant cell population. The secondary giant cells are morphologically indistinguishable from the primary giant cells; are formed in the periphery of the EPC and spread laterally and ventrally to completely surround the conceptus and its membranes. Studies by Rossant et al. (1978) supported the evidence that the extraembryonic ectoderm also originated from the polar trophectoderm.

The ICM gives rise to the primitive endoderm which following its expansion at the periphery over the inner surface of the

trophoblast, becomes the parietal endoderm layer (Snell and Stevens, 1966). The proximal (or visceral) endoderm of the 5 1/2 day mouse embryo overlying both the embryonic and extra-embryonic ectoderm subsequently contributes to the endoderm of the visceral yolk sac (Gardner and Papaioannou, 1975), while the parietal (distal) endoderm cells are involved in the formation of Reichert's membrane (Enders et. al., 1978).

1.3.4 Histology of the Feto-maternal Interface

1.3.4.1 Fetal Component

In the mouse placenta, chorionic tissue (trophoblast cells with their underlying fetal capillary and stromal cells, i.e., fibroblastic and phagocytic cells, derived from extra-embryonic mesoderm) appears as interconnected strands forming a sponge-like network lining the maternal blood sinusoids. On the basis of the extent of sinusoidal surface area and trophoblast cell formation, the mature placenta is subdivided into three trophoblast zones (Billington, 1975) (Figure 3 reproduced from Chatterjee - Hasrouni and Lala, 1979).

1) The labyrinthine zone, is the largest zone closest to the embryo and contains the most extensive maternal sinusoidal spaces. The labyrinthine trophoblast cells are usually uninucleated and their attenuated cytoplasmic processes line the sinusoids. Electron micrographs of these cells demonstrate that they form three layers and thus are "trichorial" (Enders, 1965).

2) Overlying the labyrinthine trophoblast cells and closest to the decidua are the spongiotrophoblast cells which make up the spongiotrophoblast zone. This zone is characterized by large and more

rounded trophoblast cells often multinucleated and contains fewer sinusoidal spaces. Some spongiotrophoblasts extend as columns into the labyrinthine zone, and also extend to the maternal decidua (Fig. 3).

3) Giant trophoblast zone is a thin, often incomplete, layer of gigantic trophoblast cells located between the spongiotrophoblast zone and maternal decidua and runs along the periphery of the placenta all the way down to the yolk sac. Part of this layer remains in contact with the Reichert's membrane of the yolk sac. Thus all three types of trophoblast cells mentioned above constitute the fetal components of the fetomaternal interface in the mouse placenta.

1.3.4.2 Maternal Component

The maternal counterpart of the feto-maternal interface are the blood cells in maternal sinusoids and cells of the decidual tissue. Decidual tissue is composed in man and mouse of typical stromal-type decidual cells and variable numbers of immigrant cells, e.g., blood leucocytes, macrophages and granular metrial gland cells. The number of immigrant cells is usually low during early pregnancy and rises during late pregnancy (Kearns and Lala, 1985a).

Decidual cells are hormonally induced end products of endometrial stromal cells initially located on the antimesometrial aspect of the lumen (side on which the blastocyst implants in rodents) after the implantation of the blastocyst on day 4 in mouse and day 5 in the rat. This is the primary decidual zone. This zone expands and reaches a peak of development by day 9 in the mouse and then starts regressing.

FIGURE 3

A diagrammatic representation of a cross-section through the murine placenta (courtesy of Saswati Chatterjee-Hasrouni, reproduced from Ph.D. thesis, The Immunobiology of Feto-Maternal Relationships, 1981).

D - Decidual tissue

SP - Spongiotrophoblast cells. Note cords of spongiotrophoblast cells extending into the labyrinthine zone

L - Labyrinthine zone. Note the numerous sinusoidal spaces

GC - Trophoblast Giant Cells

L
D
SP
GC

Decidualization extends laterally (Decidua Lateralis) and then becomes evident on the mesometrial aspect where EPC trophoblast cells make contact with the luminal epithelium (day 7 in the mouse). When the placenta develops, this zone is the "decidua basalis" (Krehbiel, 1937). As this zone grows to a peak, the antimesometrial decidua has regressed to a thin "decidua capsularis." Decidua basalis is the major maternal component of the fetomaternal interface.

The origin of the precursors of typical decidual cells was studied by examining radioautographically the H-2 phenotype of decidual cells in pseudopregnant bone marrow chimeras (Kearns and Lala, 1982). Chimeras were produced by repopulating lethally irradiated parental strain mice with F1 strain bone marrow. Results revealed a strong correlation of the extent of chimerism between typical decidual cells and known marrow derived elements such as decidual macrophages and splenic lymphocytes, indicating that decidual cells in these animals were ultimate descendants of the bone marrow. It has further been shown that typical decidual cells, appearing during pregnancy, share certain surface markers such as Thy-1 and FcR and mac-1 with lymphomyeloid cells but lack markers such as surface immunoglobulin (Ig), I-A, I-J, Lyt (Kearns and Lala, 1984). These investigators have also identified unique tissue specific surface markers on decidual cells, recognized by monoclonal antibodies raised in this laboratory (Kearns et al, 1985 a,b). Based on these studies, decidual cells can now be listed amongst other immunoregulatory stromal type marrow derived cells such as epidermal Langerhans cells, epidermal dendritic cells and dendritic reticular cells of the lymphoid organs. The immunoregulatory properties of decidual cells will be summarized later.

1.4 Mechanisms Proposed to Explain the Nonrejection of the Allogeneic Feto-Placental Unit

The mechanisms proposed may be operating at the level of the graft (conceptus), host (mother) or graft-host junction (fetomaternal tissue interface).

1.4.1 Mechanisms Related to the Graft or the Conceptus

The conceptus constitutes three elements, a) the embryo b) the fetal membranes and c) the placenta. The question of antigenicity and/or immunogenicity of each of these components will be discussed.

1.4.1.1 The Embryo as an Allograft

Both major and minor histocompatibility antigens have been detected on the cell surface of post-implantation embryonic tissues from midgestational embryos (Edidin, 1972; Jenkinson and Billington, 1977; Kirkwood and Billington, 1981). These embryos are considered antigenic because they possess histocompatibility antigens. Immunogenicity refers to the ability of these cells to evoke a cellular and humoral immune response against the foreign surface antigens. The immunogenicity of post implantation embryonic tissues has been clearly demonstrated by the exchange of grafts between congenic strains (Patthey and Edidin, 1973) and by grafting embryos under the kidney capsule in semiallogeneic strains of mice (Simmons and Russell, 1962).

As far as preimplantation embryos are concerned, the findings are varied. Using a cytotoxicity assay, Heyner (1980) observed that early morula stage embryos expressed both H-2 and non-H-2 antigens. Some

investigators observed positive labeling of H-2 antigens on both the early and late blastocysts (Muggleton-Harris and Johnson, 1976) whereas others observed negative labeling (Hakansson et al., 1975). These conflicting results may have been derived from variable specificity of the antibodies and the sensitivity of the techniques employed. For this reason, this problem was reinvestigated in this laboratory (Lala et.al., 1984). The presence of paternal type H-2 antigens were examined on murine blastocysts at different stages of development using a highly sensitive radioautographic technique following the application of radioiodinated monoclonal antipaternal H-2 K antibody. This study showed the presence of paternal type H-2 antigens on the outer trophoblast cells of the late morula and early blastocyst, but these antigens were not detectable on the late or preimplantation blastocyst. These findings are in agreement with Billington et al. (1977) reporting the lack of antigenicity of the conceptus at the time of implantation.

1.4.1.2 The Fetal Membranes as an Allograft

In the mouse, the yolk sac membrane is a fetal membrane which comes into direct contact with the uterus during and following implantation (Theiler, 1972). In the human, the amnio-chorionic membrane makes the contact with the uterus. In both mouse (Parr et al., 1980) and man (Hsi et al., 1982) the fetal membranes do not evoke a maternal immune response because they do not express class 1 or class 2 antigens. Thus, extraembryonic fetal membranes appear to be both antigenically and immunologically inert.

1.4.1.3 **MHC Antigenicity and/or Immunogenicity of Trophoblast Cells.**

On the basis of classic studies where 2-8 cell tubal eggs, 3 1/2 day-old blastocysts and EPC were transplanted beneath the renal capsules of specifically presensitized allogeneic mice, Simmons and Russell(1962) concluded that transplantation alloantigens were present in the embryos but that trophoblast cells represented a specialized form of embryonic cells that did not express alloantigens on their surfaces effectively for them to be rejected. Whether the antigenic determinants on the trophoblast cells were absent or masked became the focus of ample investigation.

Since the above studies, the expression of histocompatibility antigens by trophoblast cells of both murine and human placenta has been extensively studied, the results of which have been very controversial. Early studies using serological and transplantation methods, as reviewed by Edidin (1972), produced negative results in favor of Simmons and Russell's conclusions.

Using the mixed hemadsorption technique (incubation with allo-antisera followed by incubation with anti-immunoglobulin (Ig) coupled to sheep red blood cells (SRBC)), Billington et al. (1977) detected H-2 and non H-2 antigens on cultured cells of the mouse EPC. These antigens were observed between 12 to 15 days of a 21 day gestation period. Using an immunoperoxidase sandwich labeling method in mice, these same investigators did not detect major histocompatibility antigens on 7.5 day old EPC. In 1977, Sellens found minor but not major histocompatibility antigens on 4 day old mouse blastocysts outgrowth, which included the trophoblast, when using the mixed

hemadsorption assay.

Radioautographic studies using high levels of sensitivity and specificity for the expression of MHC antigens on trophoblast cells were undertaken by Chatterjee-Hasrouni and Lala (1979, 1981). Initially, collagenase-dispersed single cell suspensions of mouse placentae from allogeneic (CBA ♀ x C57BL/6 ♂ or reverse) matings were subjected to a three step sandwich labeling with nonradioactive goat anti-mouse Ig to mask native FcR bound Ig molecules, followed by monospecific anti-H-2 antibodies and then I^{125} labeled goat anti-mouse Ig. Later (Chatterjee-Hasrouni and Lala, 1981) a two step sandwich labeling was employed using monospecific or monoclonal anti-MHC antibody followed by I^{125}-labeled protein A. These studies revealed that trophoblast cells expressed paternally-derived K and D (Class 1) MHC antigens between 9 and 18 days of gestation. On days 12-16, the density of class 1 antigens was found to be equivalent to that in adult thymocytes of the same genotype, with a further 50% increase in the antigenic density on day 18. It is interesting to note, however, that these studies as well as those of Jenkinson and Searle (1979) were unable to detect the presence of Ia or class 2 antigens on the trophoblast cells using their different techniques.

Above findings by Chatterjee-Hasrouni and Lala (1979, 1981) did not exclude the possibility that these MHC class 1 antigens may be masked in situ or sequestered away from the sinusoidal face of the plasma membrane. In fact, a sequestration of H-2 antigens to the parabasal plasma membrane of the epithelial cells of the small intestine was demonstrated by Kirby and Parr (1979). To examine trophoblast antigenicity in situ, Chatterjee-Hasrouni and Lala (1982)

introduced monoclonal anti-paternal anti-H-2K antibody directly into the individual placental branches of the uterine artery. Quantitation of radioautographic silver grains revealed a selective labeling of the sinusoidal face of labyrinthine trophoblast cells in heterozygous ($H\text{-}2^{k,b}$) placentae, while those from homozygous ($H\text{-}2^{bb}$) placentae remained unlabeled. This finding indicated that paternally encoded class 1 antigens are expressed on trophoblast cells in situ and that these antigens are exposed and accessible to antibodies in the maternal blood. Spongiotrophoblast cells did not show labeling at 15 min. after intraarterial infusion of antibodies, possibly due to the poor accessability of antibodies to these cells as a result of a smaller size and number of maternal sinusoids in this region. Trophoblast giant cells were not labeled.

Extensive work has also been done on the human placenta. Studies using immunofluorescence (Faulk and Temple, 1976; Sundqvist et al., 1977), immunoperoxidase (Faulk and Temple, 1976; Sunderland et al., 1981) and mixed hemadsorption (Sundqvist et al., 1977) failed to show appreciable levels of MHC antigens on villous trophoblast cells of mature and early gestational placentae. However using a highly sensitive radioautographic technique, where single cell suspensions of collagenase-dispersed human placentae (obtained from first trimester of pregnancy) were treated with monoclonal anti-HLA-A, B, C or HLA-DR antibodies, generated the following results. There was strong labeling of cytotrophoblast cells for class 1 antigens in early gestational (5-8 week) placentae with a gradual decrease of labeling from 9-12 week gestation, and no appreciable level of class 2 antigens were detectable

on these cells. Syncytiotrophoblast cells revealed no labeling for either class of antigen (Montgomery and Lala 1983). Class 1 antigen bearing cytotrophoblast cells were localized in extravillous and interstitial sites (Sunderland et al., 1981).

These reports, in human and mouse, unequivocally demonstrated the presence of class 1 but not class 2 MHC alloantigens on trophoblast cells. The absence of class 2 molecules may explain the low immunogenicity of the trophoblast. However, the fact that a multiparous mother, who has been primed to her fetus, still does not reject the fetal allograft minimizes the role of the lack of class 2 antigens in the maintenance of pregnancy. In fact, a priming of the female with allogeneic cells of the fetal genotype bearing both class 1 and class 2 alloantigens does not influence the outcome of allogeneic pregnancy (Wegmann et al., 1979 c). Furthermore, in spite of the presence of class 1 alloantigens, murine trophoblast cells are not immunogenic in vivo. Beer and Billingham (1980) suggested that the trophoblast behaves as an immunologically privileged tissue. In vivo experiments by several investigators revealed that allogeneic transplants of trophoblast at ectopic sites were nonimmunogenic as well as refractory to immunologic attack (Kirby et al., 1966; Simmons and Russell, 1962; Lanman et al., 1962; Hunt and Avery, 1976). In vitro studies on 10 1/2 day post-coitum murine placental trophoblast cells, on the other hand, have shown some detectable immunogenicity (Pavia et al., 1981) as well as susceptibility to killing by specifically primed T cells (Smith, 1983). However, a major criticism of these studies is that contamination of other antigenic fetally derived cells in the trophoblast cell preparation may have biased the results. How

trophoblast cells escape immunological destruction is unclear at present and since failure to express appropriate target antigens cannot be the reason for the in vivo survival of trophoblast cells, it must be presumed that some form of immunoregulatory mechanism(s) is responsible for the maintenance of the fetoplacental allograft.

1.5 Mechanisms Related to the Mother (Host)

1.5.1 Uterus as an Immunological Privileged Tissue

A site is considered immunologically privileged if it lacks lymphatic channels and is hence quarantined from the lymph nodes where the sensitization against the allograft may occur. Some examples are the brain, the anterior chamber of the eye and the cheek pouch of the hamster. In 1968, Kirby proposed that the uterus was a privileged site because allografts placed in close apposition to developing trophoblast or decidual bed during pregnancy survived for prolonged periods. However, this hypothesis can now be discarded not only because the uterine endometrium has been found to have a good lymphatic drainage (Head and Billingham, 1981) but also because following its sensitization to allogeneic skin grafts, there is a second immune response generated resulting in the prompt rejection of the graft (as reviewed by Beer and Billingham, 1976). Furthermore, in the absence of pregnancy, Currie (1970) observed that allogeneic skin grafts in one uterine horn incited a transient hypertrophy of the draining lymph nodes.

1.5.2 Systemic Immunosuppression in the Mother

During pregnancy there is clinical and experimental data which

indicates a weak suppression of the mother's immunological reactivity. This is due to (1) the release of some nonspecific immunosuppressants such as pregnancy associated proteins, steroid and protein hormones produced by the placenta and (2) systemic suppressor cells in the pregnant mother.

1.5.2.1 Pregnancy Associated Proteins

Early pregnancy factor (EPF) is a pregnancy associated protein which is found in the serum of humans and mice within hours of fertilization of the egg (Morton et al., 1974). EPF inhibited the rosette formation in vitro (Bach and Antoine, 1968) and prolonged the survival of allografts in vivo (Munro et al., 1971).

Another pregnancy protein, probably produced by peripheral blood leucocytes is pregnancy zone protein (PZP) (Stimson et.al., 1977). PZP was found to suppress the migration of leucocytes to Bacille Calmette-Guerin (BCG) in vitro (Stimson and Blackstock, 1976) and also the response of peripheral blood leucocytes to phytohemagglutinin (PHA) (Stimson, 1980).

Another candidate protein is β_1-glycoprotein which suppressed the blastogenic response of phytohemagglutinin induced peripheral blood lymphocytes in vitro (Smart et al., 1981).

1.5.2.2. Peripheral Suppressor Cells

An examination of peripheral T lymphocyte subsets in allogeneically pregnant mice shows an increase in the subsets bearing Ly 2, 3 and I-J phenotypes possibly indicative of some suppressor function (Lala et al. 1983 a). Chaouat et al. (1977, 1979, 1980)

reported the generation of systemic suppressor T cells in multiparous pregnant mice using a number of in vivo and in vitro functional assays. They observed that the suppressor cells had Lyt 2^+, Ia^+ and I-J^+ phenotype and they displayed MHC restriction. Nagarkatti and Clark (1983) have reported specific suppressor T cells (Thy 1.2^+, Lyt 1^-2^+) that are distributed systemically in peripheral lymph nodes and spleen after a single allogeneic pregnancy in C3H and A strain mice. These suppressor cells inhibited the generation of cytotoxic cells against paternal alloantigens in vitro. In vivo, these suppressor cells enhanced the growth of intrauterine innocula of allogeneic paternal haplotype P 815 tumor cells in pseudopregnant compared to control animals.

The systemic immunosuppression resulting from the production of immunoregulatory factors and systemic presence of T suppressor cells in the mother during pregnancy cannot be considered to protect the fetus in vivo because the mother can easily reject tissue grafts from her semiallogeneic progeny during pregnancy without any harm to the fetoplacental unit (Woodruff, 1958). The appearance of suppressor T cells may indicate a normal homeostatic response of the mother in recognition of the alloantigens of the fetoplacental unit.

1.6 Mechanisms Related to the Graft-Host Junction (Fetomaternal Interface)

1.6.1. Masking

1.6.1.1. THe Fibrinoid Theory

This hypothesis contends that the trophoblast cell surface is

masked with an amorphous layer of electron dense fibrinous material of sulfated sialic acid rich mucoprotein (Bardawil and Toy, 1957; Bradbury et al., 1965). Currie and Bagshawe (1967) proposed that the fibrinoid layer exerted its immunoprotective function by covering the trophoblast's antigenic determinants, or by producing a negatively charged electrostatic barrier which would repel similarly charged maternal lymphocytes. However, this hypothesis is refuted by several studies:

(1) Trophoblast immunogenicity reported after neuraminidase treatment (Currie et al., 1968) has not been confirmed by later studies of Searle et al. (1975).

(2) Martinek (1970, 1971) observed that there is an incomplete fibrinoid layer at the trophoblastic decidual tissue interface of the human and rat placenta.

1.6.1.2 Masking of Trophoblast Antigenic Sites by Other Molecules

It has been postulated that transferrin receptors found on the human syncytiotrophoblast plasma membrane (Faulk and Galbraith, 1979a, 1979b) may act as an antigen masking agent. Another molecule considered as a masking agent is uteroglobin (Mukherjee, 1982). It is proposed that with the help of the enzyme transglutaminase uteroglobin may cross link β 2 microglobulin, the light chain constituent of the class 1 MHC antigen, and hinder antigenicity of trophoblast cells. While uteroglobin has been identified in only few species, it is of interest that a deficiency of transglutaminase results in pregnancy failure in women (Fisher et al, 1966). A similar masking role has also been assigned to hCG, a product of trophoblast cells (Beer and

Billingham, 1979). This hormone is believed to produce a highly negative charge on the trophoblast plasma membrane (Wrezlewicz et al., 1977) causing an electrostatic repulsion of lymphocytes.

1.6.1.3 Role of Blocking Antibodies in the Protection of the Fetus

Blocking antibodies are non-cytotoxic classes of antibodies which combine with the antigenic sites on target cells and prevent them from further sensitizing the host and from being recognized by any sensitized effector lymphocytes. Their role in the protection of the fetus was initially suggested by the presence of anti-paternal strain agglutinins in the circulation during murine pregnancy (Kaliss and Dagg, 1964). Blocking antibodies eluted from midterm heterozygous murine placenta have been reported to enhance the growth of paternal strain sarcoma in an antigen specific manner (Voisin and Chaouat, 1974). Taylor and Hancock (1975) demonstrated that maternal lymphocytes could be activated to killer cells in the presence of trophoblast cells in vitro. But the killing was abrogated with the addition of maternal serum and restored with the removal of serum IgG.

Several reports have detected a variation in the properties of these pregnancy induced alloantibodies; in the human, horse and cow they include cytotoxic antibodies whereas in the mouse and rat they appear to be non-cytotoxic in nature (Bell and Billington, 1981; Smith et al., 1982). These antibodies are usually restricted to the IgG_{2a} subclass in the rat (Ghani et al., 1984), in the mouse serum (Bell and Billington, 1980), as well as placental eluates (Voisin and Chaouat, 1974). In both rat and mice the antibody response was shown to be

against the MHC or H-2 and to some extent to non-H2 antigens in the mouse (Ghani et al. 1984; Bell, 1984). Recently, Bell (1984) studied seven female mice strains, and only three, all H-2^b haplotype strains were responders after a third pregnancy; i.e., produced antipaternal alloantibodies. The remaining four strains produced no detectable alloantibodies as measured by mixed hemadsorption assay, even after six pregnancies with male strains differing at combined H-2 and non H-2 loci.

The absence of antibodies against paternal antigens in some but not all strains of mice (Bell, 1984) and rat (Ghani et al., 1984) despite the presence of MHC differences between mother and fetus, leads to the conclusion that blocking antibodies are not necessary for the survival of the fetoplacental unit.

1.6.2 The Local Immunoregulation by Cells at the Fetomaternal Interface

Since the findings on trophoblast antigenicity or the systemic immune status of the mother cannot explain the survival of the fetoplacental unit, the key mechanism of protection must be sought at the fetomaternal interface. In support of this contention are the findings of Beer and Sio (1982) who demonstrated that skin allografts at the choriodecidual junction survive for longer periods. The immunoprotective role may be exerted by the fetal and/or maternal components at the fetomaternal interface.

1.6.2.1 Fetally Derived Cells and Their Products

The important role of trophoblast cells has achieved more

attention by the findings of Rossant et al. (1982) who showed that trophoblast phenotype determines the survival of chimeric embryos produced by blastocyst aggregation. Besides possibly unique surface membrane properties of trophoblast cells (Chatterjee-Hasrouni and Lala, 1982), these cells may also exert a local immunoregulatory role. Several immunosuppressor hormones are secreted by these cells such as progesterone, hCG and human placental lactogen (hPL), all of which are found at much higher concentrations in the placenta than in the blood (Siiteri et al., 1982). Of these, progesterone remains as the most viable candidate. However, trophoblast cells may produce other undescribed immunosuppressor molecules (Pavia and Stites, 1981).

Trophoblast cells may play an indirect role in local immunosuppression (Clark et al., 1983) by recruitment of local nonspecific uterine suppressor cells described by Slapsys and Clark (1982). These investigators believe that during interspecies mouse pregnancy fetal death occurs due to a breakdown of the fetomaternal barrier because the trophoblast cells have failed to recruit suppressor cells.

1.6.2.2 Maternally Derived Cells and Their Products

Maternal cells which may have immunoregulatory functions are decidual cells and decidual leukocytes (lymphocytes and macrophages) which constitute the decidua. In vivo studies by Beer and Billingham (1974) detected that allogeneic skin grafts in decidualized pseudopregnant uteri survived significantly longer than those in nondecidualized uteri. However, skin grafts were promptly rejected

when introduced into specifically primed animals. These findings suggested that the afferent arm and not the efferent arm of the immune response was compromised by the decidua. More recently Sio et al (1983) extended these studies to show that skin allografts can be made to survive indefinitely if implanted at the choriodecidual junction,strongly indicating a local immunoregulation by the decidua.

An immunosuppressor class of lymphocytes in the pregnant endometrium and lymph nodes draining the uterus in primigravid mice has been reported by Clark et al (1978) and Slapsys and Clark (1982). They are small granulated lymphocytes devoid of T cell markers. These cells inhibited the generation of cytotoxic T cells against paternal alloantigens in vitro at time of implantation and again during the second half of pregnancy. Decidual cells have been reported to suppress multiple immune responses of lymphocytes in vitro. They were found to suppress the antibody response of normal adult spleen cultures to Dinitro-phenol (DNP) polylysine (Globerson et al., 1976) and proliferative response of lymphocytes to allogeneic cells (Golander et al. 1981). In addition, the proliferative phase of the mixed lymphocyte reaction (MLR) was inhibited by decidual cells (Lala et al., 1983a) and their supranatants (Kirkwood and Bell, 1981). In the human, purified decidual cells obtained from early placentae (5-12 weeks) suppressed the MLR and generation of cytotoxic T lymphocytes in an MHC-unrestricted manner (Parhar and Lala, 1985). Similarly, decidual cells and decidual macrophages were found to be capable of suppressing the generation of killer activity in the natural killer lineage lymphocytes migrating into the murine decidua (Scodras et al 1985), which recognize

target structure on murine trophoblast cells (Chatterjee-Hasrouni et al., 1984).

Thus, decidual cells and immigrant leucocytes in the decidua appear to play a local immunoregulatory role.

1.7 The Placenta as an Immunological Barrier

1.7.1 Separation of Maternal and Fetal Circulations

In every species studied there is no direct vascular communication between the maternal and fetal circulations in the placenta. The importance of this separation was demonstrated by Scott and colleagues (1973). They surgically anastomosed maternal to fetal vascular compartments in the placenta, bypassing the dialysis membrane of the trophoblast, in the rats, rabbits and guinea pigs. It was found that, irrespective of the degree of genetic compatibility, the fetus would die of blood overload within a few days. Siiteri et al. (1977) and Beer and Billingham (1979) suggested that progesterone produced by the trophoblast cells may prevent the fetal capillaries from penetrating the trophoblastic tissue and linking up with the maternal sinusoids.

1.7.2 Placenta as a Barrier to Maternal Cells

The placenta acts as a general barrier to cellular traffic in either direction, but trophoblastic tissue may gain access to the maternal blood.

In man, and certain other species, multinucleate masses of syncytiotrophoblast may break free into the maternal circulation. By the 26th day of gestation onward in man, trophoblastic emboli have been observed in the lungs, where they disappear without evoking an

immunological response (Adinolfi, 1975)

Passage of fetal erythrocytes into the maternal circulation resulting in Erythroblastosis Fetalis is known to occur primarily during a second pregnancy (Chown, 1954) most likely due to a passage of fetal red cells to the mother following placental separation. Transfer of red blood cells in the reverse direction has been identified in the human both during pregnancy and at delivery (Zipursky et al., 1963, Woodrow et al., 1971).

Leucocyte traffic from the fetus to the mother during murine pregnancy has been demonstrated by several investigators using MHC antigens (Collins et al., 1981) or sex chromosomes as markers (Adinolfi, 1975) but the results have not been reproducible by other investigators (Reviewed by Adinolfi, 1975).

Leucocyte traffic from the mother to the fetus across the placental barrier is a rare phenomenon (Weggmann, unpublished data), although reported on occasion using cytogenetic markers (Turner et al, 1966). Generally, the consequence of such an event is runt disease and death of the fetus.

Thus, the placenta normally acts as a barrier to cellular traffic in both directions but more importantly from the mother to the fetus.

1.7.3. The Placenta as an Immunoadsorbent Barrier

This hypothesis was first proposed by Swinburne (1970) to explain how the placenta protects the fetus from potentially harmful anti-paternal (or antifetal) antibodies.

During pregnancy, the mother is cognizant of her alien

fetoplacental unit and produces maternal antibodies, which are more evident in multiparous pregnancies (Bell and Billington, 1981), directed against the fetal MHC antigens. Antipaternal type antibodies have been detected in the placental eluates (Tongio et al., 1975; Doughty and Gelsthorpe, 1974; Jeannet et al., 1977) some of which are cytotoxic (Taylor, 1973). On the other hand, similar antibodies have not been demonstrable in the blood of the newborn in several species, e.g., man (Doughty and Gelsthorpe, 1974) and rabbits (Lanman, 1965). This would support the role of the placenta in protecting the fetus from antifetal antibodies.

Experiments from the laboratory of T.G. Wegmann and his colleagues (Wegmann et al., 1979a, 1979b, 1980; Raghupathy et al., 1981) were designed to test this hypothesis. Female mice bearing heterozygous and homozygous placentae at day 13 and 17 of gestation were injected intravenously with a radiolabeled anti-paternal type polyclonal (Wegmann et al., 1979a) or monoclonal (Raghupathy et al., 1981) H-2 antibody. The animals were sacrificed from 2 hours onwards and the retention of radioactivity per gram of placenta and fetus was calculated. They found a tenfold increase in antibody bound to the allogeneic placenta which bears the target H-2K antigen, when compared to the syngeneic placenta lacking the specific antigen. Similar results were also obtained using the $F(ab)_2$ portion of the monoclonal antibody indicating that the selective retention was primarily due to antigen-antibody binding. The peak binding was noted at 8 hours. It was suggested that the cells within the placenta possessing paternally-derived class 1 MHC antigens provided an immunoadsorbent

role. However, no preferential retention of the antipaternal type class 2 MHC antibodies was seen. This suggested a lack of class 2 MHC antigen-bearing cells at the fetomaternal interface (Raghupathy et al., 1981).

These authors contended that the immunoadsorbent function of the placenta was likely exerted by the trophoblastic epithelium (Wegmann, 1981) primarily belonging to the spongiotrophoblast zone (Singh et al., 1983). Although some immunochemical evidence of antibody breakdown within the placenta was presented (Raghupathy et al., 1984), the exact histological localization of the initially bound antibody and its ultimate pathway in the murine placenta remained undetermined in these studies.

OBJECTIVES

1 **To establish the identity of various fetally derived cells within the 15d murine placenta, which express in situ the class 1 MHC antigens of the paternal haplotype.**

Previous studies in this laboratory (Chatterjee-Hasrouni and Lala, 1982) identified labyrinthine trophoblasts as the antigen-bearing cells from their binding to antipaternal type H-2K antibody in situ at 15 minutes after a single injection of the antibody via the placental artery. Although labeling of spongiotrophoblast and trophoblast giant cells was not detectable with this protocol, this study did not exclude the possibility that the negative findings may have resulted from a poor access of the antibody due to a paucity of sinusoids in these areas and that a longer interval may be needed for the antibody to reach these sites. For this reason, the present study followed the specific in vivo labeling pattern of various cells within the placenta at frequent intervals for a longer time period (2, 5, 15, 30 min, 1 hour and 2 hours) after a single intravenous injection of antipaternal type monoclonal H-2K antibody. This was carried out with the application of quantitative radioautography to tissue sections at the light microscope level. A systemic (intravenous) rather than a local (intraarterial) route was chosen by necessity to keep the animal alive for a longer duration of the experiments.

2 **To identify tentatively the cells in the 15d murine placenta which may perform the immunoadsorbent function in limiting the passage of antifetal type H-2 antibodies from the mother to the fetus.**

Wegmann and his associates (Wegmann et al., 1979a, 1979b, 1980; Raghupathy et al., 1981) have demonstrated the above specified function of the placenta at 13-17d of gestation in another mouse strain from a selective retention of radioactivity by the heterozygous placenta after an intravenous injection of radiolabeled antibodies. However, their studies were confined to longer time intervals (2-24 hr) after the antibody injection. It is possible that during the first two hours the specifically bound antibody may have already been processed by the cells in the placenta. For this reason the current study was performed at earlier time intervals to evaluate whether (a) there was a higher uptake of the radioactivity by the heterozygous placenta than the homozygous placenta at these intervals and (b) such an uptake represented a selective immunoadsorbent function of the placenta in preventing the entry of harmful antifetal antibodies into the fetal circulation. This was investigated by a measurement of the whole placental radioactivity relative to that of the fetus in allogeneic and syngeneic pregnancy. Although Wegmann and his associates have suggested that spongiotrophoblast cells perform the immunoadsorbent function (Singh et al.,1983) a precise morphological identity of the cell type(s) which play this role remains to be established more firmly. The present study has attempted to identify those cells at the light and electron microscope level.

3 To explore the role of Fc receptors (which are abundant in most cells of the placenta) in the binding of IgG class antibodies in general, as opposed to H-2 related binding of such antibodies.

This was done from an evaluation of (a) in vivo retention of radiolabeled unrelated anti-H-2K^k antibody within the homozygous C57BL/6 placenta used as controls (b) in vivo retention of radiolabeled unrelated anti-HLA-DR antibody within (C57BL/6♀ x C57BL/6♂) and (C57BL/6♀ x CBA♂) placentae (c) in vitro labeling of placental cells with radiolabeled anti-H-2K^k and anti-HLA-DR antibodies under different labeling conditions (e.g. at different temperatures; in the presence of excess of IgG, or non radioactive H-2K^k antibody).

4 To examine at the ultrastructual level (a) the localization of H-2 antigenic sites on the trophoblast plasma membrane and (b) the subsequent movement of the label after the initial binding of the radiolabeled antibody to the plasma membrane.

Previous studies in this laboratory (Chatterjee-Hasrouni and Lala 1982) have demonstrated a preferential localization of the radioactivity in situ on the sinusoidal face of labyrinthine trophoblast cells at the light microscope level at 15 min after an intraarterial injection of radiolabeled antipaternal type H-2K antibody. The current study, at the ultrastructural level, is an extension of this work to examine whether H-2 antigenic sites are indeed located on the sinusoid lining plasma membrane of the labyrinthine trophoblast, and whether other fetally derived cells (inclusive of other trophoblast cell types) exhibit specific labeling of the plasma membrane. Finally, an attempt was made to characterize the immunoadsorbent function of the placenta by an ultrastructural follow up of the labeling sites of those cells which were found to show a strong retention of the labeled antibody at the light microscope level.

PART 2 MATERIALS AND METHODS

2.1 Mice

Inbred C57 BL/6J females ($H\text{-}2^b$) and CBA/6 males ($H\text{-}2^k$) mice (8-20 wks of age) were obtained from Jackson Laboratories (Bar Harbor, Maine). C57 BL/6J ♀ mated with CBA/6 ♂ (b x k) provided the allogeneic matings whereas the C57 BL/6 J ♀ mated with C57 BL/6 J ♂ (b x b) provided the syngeneic matings. Pregnant mice were sacrificed at day 15 of gestation. The day of appearance of a vaginal plug was counted as day 0 of pregnancy.

2.2 Antibodies and Immunoglobulin

Monoclonal anti-H-2 k^k (IgG_{2a}) antibody (1 mg/ml; Becton Dickinson and Co., Mountainview, California) was used for these experiments. This antibody has been previously characterized (Oi et al., 1978). Monoclonal anti-HLA-DR (Lampson and Levy 1970; Clone L243; Heavy chain IgG_{2a}) was purchased from Becton Dickinson, Sunnyvale, CA. Affinity purified mouse IgG (5 mg/ml) was obtained from Cedarlane Laboratories Ltd., Hornby, Ontario.

2.3 Radioiodination

Antibodies were coupled to carrier free $Na\text{-}I^{125}$ (specific activity 1.5×10^7 μCi/mg, concentration 100 mCi/ml; Charles Merck Frosst Co., Montreal, Que.) by a modification of the chloramine T method of Greenwood et.al., (1963).

The affinity purified monoclonal antibodies were mixed in 25 μg (in 25 μl) aliquots on ice with 1.1 mCi (11 - 15 μl) $Na - I^{125}$ and coupled by the addition of 5 μl chloramine - T (2 mg/ml in distilled

water (d H_2O); Eastman Kodak Co., Rochester, N.Y.). After 10 min. the reaction was stopped by adding 5 µl potassium metabisulfate (4.8 mg/ml in d H_2O ; J.T. Baker Co., Phillipsburg, New Jersey) and 20 µl 0.1 M potassium iodide.

Fractionation through a Sephadex G-25 gel filtration column (Pharmacia, Dorval, Que.) separated I^{125}-bound protein and free Na-I^{125}. The column was prepared by pouring the preswollen gel (3 g G-25) in 30 ml 0.1% Bovine Serum Albumin ((BSA; Gibco, Grand Island, N.Y.) in PBS pH 7.3, 3 hrs at room temp.))into a vertically secured 10 ml syringe, prepacked with a layer of glass wool to retain the gel. After 20 mls of 0.1% BSA-PBS ran through the gel, a cut circle of filter paper (Whatman #1) was placed on the gel surface to stabilize the gel bed. A 21 gauge needle attached to the syringe controlled the flow rate. The iodination mixture was applied to the column when the fluid level had reached the surface of the gel. Once the mixture passed through the filter paper it was eluted through the column with 0.1% BSA-PBS, pH 7.3. Thirty fractions at one minute intervals were collected and the activity in 5 µl aliquots of each tube was counted in a Gamma Counter (Gamma 4000; counting efficiency 72%, Beckman instruments, Fullerton, CA). Fractions containing the first peak of activity were pooled and stored under sterile conditions at 4°C. The specific activity yielded was between 24-44 µCi/µg, the average being 36 µCi/µg.

2.4 Perfusion Apparatus

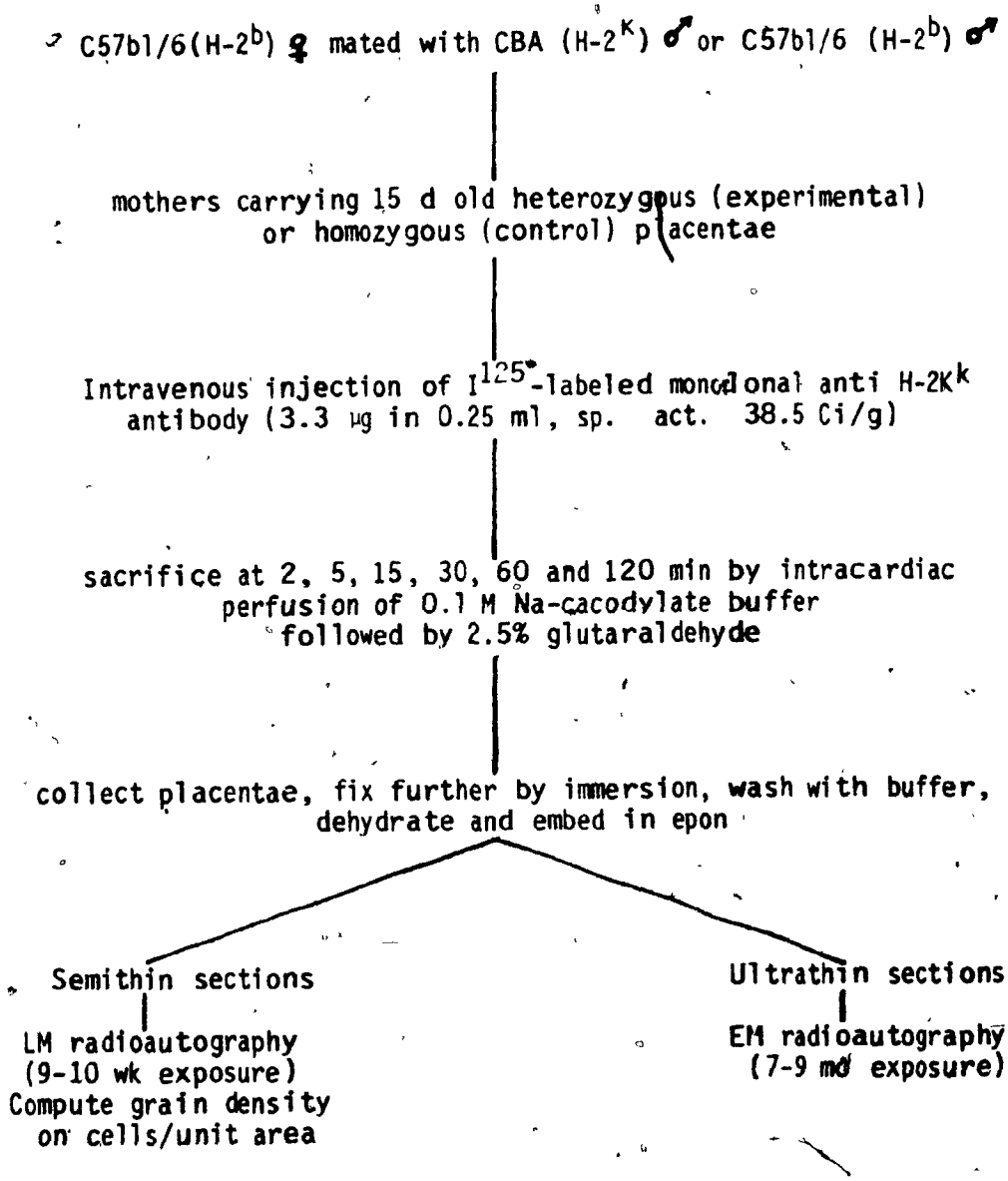
Hooked up to a T bar were two one litre infusion bottles, one filled with 2.5% glutaraldehyde in sodium cacodylate (Na Cacod.) and

the other bottle with 0.2M Na cacod. buffer only. These bottles were hung vertically with their mouths facing down connected to a "Venoset 78" infusion set (Abbott Hospitals Inc., Chicago, IL). The perfusate could be controlled to flow down the infusion line at a rate of one drop/sec by manipulating the valve. The two tubings were connected to each other at the T junction and thus fluids in both these lines would flow into the same 20 1/2 gauge needle. All air was removed from the system prior to perfusion.

2.5 Intravenous Injection of Antibody (Flow Chart 1)

All pregnant animals were warmed up with an infrared lamp for 5 min. to cause vasodilation prior to the injection of antibody into their tail vein. Each animal was injected, using a 30 1/2 gauge needle, into the lateral tail veins with undiluted monoclonal anti-H-$2k^K$ antibody. The volume varied depending on the total counts per minute (cpm) and specific activity of the pooled antibody fractions. The total protein bound activity injected into each animal ranged between 116-140 μCi (on most occasions 127 μCi) in 0.25 - 0.3 ml volume. Prior to perfusion, the mice were anaesthetized with ether. A midline ventral incision was made to expose the thoracic and abdominal cavities. At precisely 2 min., 5 min., 15 min., 30 min., 1 hr and 2 hrs after the injection of antibody, the mice were perfused through the left ventricle with 0.2M Na cacod. buffer until the placentae had visibly blanched (2-4 min.). This washing of free unbound antibodies from the placenta was immediately followed by perfusion for 15 min. with 2.5% gluteraldehyde in 0.2 M Na cacod. buffer, pH 7.3.

FLOW CHART 1


♀ C57bl/6(H-2b) ♀ mated with CBA (H-2K) ♂ or C57bl/6 (H-2b) ♂
mothers carrying 15 d old heterozygous (experimental) or homozygous (control) placentae
Intravenous injection of I125*-labeled monoclonal anti H-2Kk antibody (3.3 µg in 0.25 ml, sp. act. 38.5 Ci/g)
sacrifice at 2, 5, 15, 30, 60 and 120 min by intracardiac perfusion of 0.1 M Na-cacodylate buffer followed by 2.5% glutaraldehyde
collect placentae, fix further by immersion, wash with buffer, dehydrate and embed in epon
Semithin sections
LM radioautography (9-10 wk exposure) Compute grain density on cells/unit area
Ultrathin sections
EM radioautography (7-9 md exposure)

In a number of pregnant animals, 5 µl volumes of orbital venous blood was collected at short intervals (2 min-6 hr) after an intravenous injection of radiolabelled antibody to follow the activity of the isotope in the blood. These animals were sacrificed at 6 hrs.

2.6 Histological Preparation (Flow Chart 1)

Preparation of tissues: Following the fixation by perfusion with 2.5% gluteraldehyde, the placentae were detached from the fetus by severing the umbilical cord and removing the yolk sac and amnion; remaining was the placenta. The fetus corresponding to each placenta and a small piece of the midquadrate lobe of the liver of each pregnant mother was also removed and washed 3x in 0.2M Na cacod. buffer. These tissues were then refixed in 2.5% gluteraldehyde by overnight immersion at 4°C. The next day the tissues were washed 6x for 10 min. in Na cadod. washing buffer (100 mls 0.2M Na cacod.) blotted free of moisture, weighed and a one minute gamma count of radioactivity was obtained on the Gamma counter.

Only the placentae were processed further for light and electron microscope radioautography.

Placentae were cut into 1 to 2mm trapezoid shaped pieces allowing an orientation of the fetal and maternal sides during further processing for sections. Tissues were post-fixed for 1 1/2 hrs in 2% osmium tetroxide containing 1.5% ferrocyanide (1:1 v/v 4% osmium tetroxide : 0.2M ferrocyanide at 4°C). They were then dehydrated by immersion in increasing concentrations of ethanol (2 x 5 min.: 50%, 70%, 80%, 90%, 95% ethanol, 3 x 10 min. 100% ethanol). Dehydrated tissues were then passed through 100% propylene oxide for 20 minutes. Subsequently, they were infiltrated with increasing concentrations of Epon (Epon 812 17g, dodecenyl succinyl anhydride 10g, nadic methyl anhydride 8.6g and 0.8 mls DMP-30 catalyst) by immersion in epon-propylene oxide mixtures of 1:1 v/v overnight, 2:1 overnight and

pure epon (free from bubbles) overnight. Tissues were embedded using the cone headed capsules and removed from a 60°C oven after 72 hrs. of polymerization. Each block was later trimmed to be cut.

2.7 Light Microscope Preparations

Semithin (0.5 μm) sections of the blocks were cut using glass knives on a Reichert OM-US ultramicrotome (Reichert, Austria). Sections were floated on d H_2O and transferred onto a clean glass slide. Five sections were placed on each slide which was then heated at 80°C on a hot plate for 1/2 hr to make the sections adhere. Prior to radioautography, these sections were stained with iron alum hematoxylin. Slides placed on hot plate maintained at 80-85°C were covered with iron alum (5% aqueous ferric ammonium sulfate) for 15 min. They were then washed with d H_2O and stained with Regaud's Hematoxylin for 5-10 min., washed and differentiated with tap water for 3 min., rinsed and air-dried.

2.8 Electron Microscope Preparations

Ultrathin sections were cut with a diamond knife on a LKB-Huxley ultramicrotome (LKB Instruments, Inc., Rockville, MD). Three groups of sections were placed on slides coated with 1% (v/v) celloidin in isopentyl acetate. Slides were carbon coated prior to radioautographic processing.

2.9 Light and Electron Microscope Radioautography

For light microscopy, sections mounted on slides were coated with Kodak NTB-2 nuclear emulsion (Eastman Kodak Co., Rochester, N.Y.) and processed by the method of Kopriwa and Leblond (1962). All definite data was derived from a 60 day exposure time.

For electron microscopy, slides containing carbon coated sections were coated with Ilford L-4 emulsion (Ilford Ltd., Ilford, Essex, England) and processed by the method of Kopriwa (1973). Exposure times ranged between 7 and 10 months. Development was carried out for large filamentous grains with Kodak D 198 chemical developer. Following development and fixation, the sections were lifted and transferred to 300 mesh copper grids. These grids were then immersed in glacial acetic acid for 3 min. to remove the celloidin and then post stained in aqueous 4% uranyl acetate for 5 min., washed in double d H_2O, stained in Reynolds lead citrate for 2 min., washed, and dried on filter paper. They were examined under either a Siemens 1 A or JEOL JEM 100 CxII electron microscope.

2.10 Analysis of Radioautographic Preparations

Placental sections were scored under the light microscope using an eyepiece micrometer grid. Using a magnification of 1000x, grains were counted per unit area of 76 μm^2. The results are expressed as the absolute number of grains per unit area of various cells without the subtraction of background. At least 1000 unit areas of 76 μm^2 were scored over the trophoblast cells. For other cell types, decidual lumen and fetal endothelium, 300 to 1000 areas were scored. Data was derived from 6 placentae per interval each pertaining to syngeneic or allogeneic pregnancy.

Electron microscope radioautographs were evaluated qualitatively. That is sections were scanned and the localization of silver grains within murine placentae was documented.

2.11 Preparation of Single Cell Suspensions

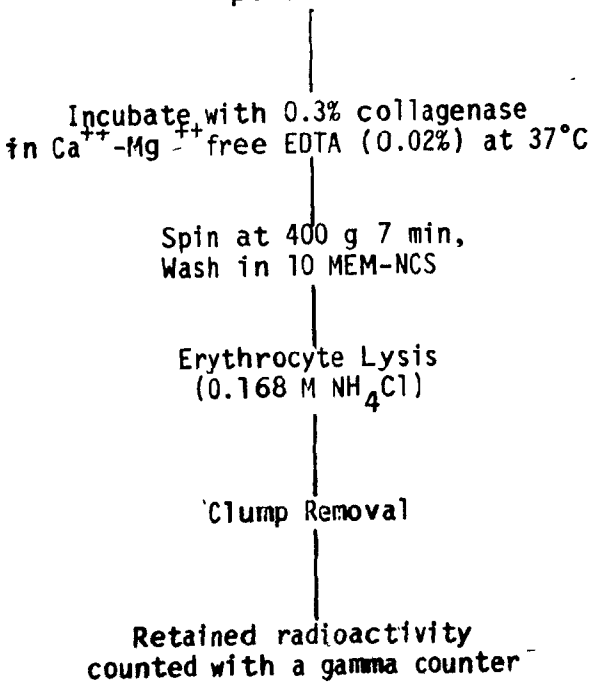
2.11.1 Spleen

The organ was removed, rinsed with ice-cold minimum essential medium (MEM, Grand Island Biological Co., Grand Island, New York) containing 10% newborn calf serum (10% MEM-NCS), minced with a pair of Dewicker's Iris scissors and gently passed through a stainless steel screen (80 mesh/inch^2). The cell suspension was centrifuged at 400g for 7 min. The supernatant containing debris was discarded and the pellet was resuspended in 10% MEM-NCS. One ml NCS was layered underneath the resuspended cells and allowed to stand at 4°C for 5 min. Cell clumps and debris settled below the interface. The top layer, free of debris, was collected, centrifuged at 400 g for 7 min. and the pellet was resuspended in 10% MEM-NCS for cell and gamma counts.

2.11.2 Placenta (Flow Chart 2)

Fifteen day old placentae were removed, rinsed and minced into small pieces with Iris scissors. They were incubated at 37°C in approximately 50 mls of 0.3% collagenase (Sigma Chemical Co., St. Louis, Mo.) made up in Ca^{++}-Mg^{++} free phosphate-buffered saline(PBS pH 7.4) containing 0.02 % disodium ethylene diamine tetracetate for 45 min. in a 37°C shaking water bath. The dispersed cells were then centrifuged at 400 g for 7 min. The supernatant was removed, the pellet was resuspended in 0.168 M NH4Cl for red cell lysis, layered over 1 ml of NCS for clump removal and centrifuged as before. This wash through NCS was repeated twice more. The final pellet was resuspended in 0.5 mls of 10% MEM-NCS and after a cell count (in the

FLOW CHART 2


Mince
15 day heterozygous and homozygous placentae
Incubate with 0.3% collagenase in Ca^{++}-Mg^{++} free EDTA (0.02%) at 37°C
Spin at 400 g 7 min,
Wash in 10 MEM-NCS
Erythrocyte Lysis
(0.168 M NH_4Cl)
Clump Removal
Retained radioactivity
counted with a gamma counter

Coulter Counter) the cell bound radioactivity was determined from a gamma count for one minute.

2.12 Experiments Testing the Specificity of Anti-H-2K^k Antibody Binding to the H-2k^k Determinants on Placental Cells.

Several experiments conducted in vivo and in vitro were designed to examine the specificity of the monoclonal antibody binding to the target paternal-type MHC antigen. Heterozygous placentae resulting from allogeneic mating should express the paternal-type H-2k^k antigen whereas homozygous placentae resulting from syngeneic mating should not bear the target antigen. Hence any preferential labeling of the heterozygous placentae over that of homozygous placentae should be due to antibody binding to the antigens, rather than antibody binding by other means eg. via Fc receptors.

2.12.1 In Vivo Binding

The overall antibody binding to the placenta was initially analysed by comparing the bulk counts of homozygous and heterozygous placentae (cpm/g) following perfusion fixation.

Labeling was further quantitated at the cellular level in light microscope radioautographic preparations of both placental types to provide information on 1) paternal-type H-2 related labeling of placental cells 2) the labeling pattern of various cell types with time and 3) possible non H-2 related labeling.

Labeling at the electron microscope level was studied to collect qualitative rather than quantitative information of the subcellular pathway of the label following the initial binding of the antibody to the cell surface H-2 antigens.

Non H-2 related binding (eg. via Fc receptors) was also tested by the intravenous injection of a mouse monoclonal antibody to human HLA-DR antigens, isotype IgG_{2a}. Mice injected with 0.23 ml (9.8×10^7 cpm equivalent of 127 μCi of radioiodinated HLA-DR were sacrificed at various time points by intracardiac perfusion in the same manner as described earlier. The placentae were washed, weighed and counted for 1 minute with a gamma counter. They were not processed for radioautography.

2.12.2 In Vitro Binding

1. Although the binding specificity of the monoclonal anti-H-2K antibody to the H-2K antigens was commercially tested, we decided to reascertain the specificity of the antibody by incubating 2×10^6 cells (in 100 μl) from the spleens of (C57BL/6 x CBA) F1 and C57 BL/6 mice with 100 μl (1μg) of monoclonal I^{125}-H-$2K^k$ at 4°C for 30 minutes. The single cell suspension was washed through several layers of NCS, centrifuged and the pellet was counted with a gamma counter. The results are presented in Table I.

2. Ten million collagenase dispersed cells from heterozygous and homozygous placentae were incubated with 100 μl (1 μg) of unrelated radiolabeled antibody, I^{125}-HLA-DR (3×10^6 cpm) at 4°C for 30 minutes. This step was designed to examine the extent of non H-2 related binding via the Fc receptor. The cells were then washed 3x, centrifuged and the radioactivity of the pellet was measured with a gamma counter.

3. Temperature during binding has been reported to affect the binding characteristics in vivo and in vitro of antibody molecules to various cells. Since all our in vivo labeling experiments were done at

TABLE I

In vitro binding of radiolabeled monoclonal anti-H-2K^k antibody to spleen cells[a]

Animal Strain	Total Bound cpm
C57 BL/6 x CBA	7225 ± 657.6
C57 BL/6 x C57 BL/6	189.5 ± 84.1

a $2x10^6$ splenocytes were incubated with $3x10^7$ cpm in 1 µg of I^{125}-anti H2K^k antibody at 4°C for 30 min.

body temperature, we compared the in vitro labeling of cell suspensions of heterozygous and homozygous placentae at two different temperatures: 4°C and 37°C. Cells were incubated with I^{125}-H-$2K^k$ antibody at these temperatures for 30 min., washed 3x and then gamma counts were taken.

4. Significant non H-2 related binding, possibly via the Fc receptor, was a common finding in our in vivo experiments. Thus, to gain some insight into the affinity of antibody binding to H-2 antigenic sites as compared to Fc receptors on the cell membrane, a displacement experiment was designed in which excess non radioactive monoclonal H-$2K^k$ was added to the incubation medium along with the I^{125} labeled H-$2K^k$ antibody. Briefly, 20×10^6 heterozygous or homozygous placental cells were incubated with 1 μg of I^{125}-H-$2K^k$ together with 10 μg of cold H-$2K^k$ at 4°C for 30 min. The control experiments were substituted with equal volumes of 10% MEM-NCS. These cells were washed and then the radioactivity was counted in a gamma counter.

Similar experiments were also conducted as above using excess mouse IgG (5mg/ml) at 4°C and 37°C to evaluate the influence of this procedure on antibody binding to heterozygous or homozygous placental cells at these two temperatures. In this experiment 20×10^6 placental cells were first incubated with 38 μl (188 μg) of mouse Ig at 4°C or 37°C for 30 min., after which the cells were washed through few layers of NCS and then exposed to 100 μl (1 μg = 20×10^6 cpm) of I^{125}-H-$2K^k$ antibody at 4°C or 37°C. The cells were later washed and prepared for gamma counts.

2.13 Statistical Evaluation

Significance of differences in the mean values were evaluated with the students two-sample t-test (Spiegel, 1961) calculated by a Carl Zeiss videoplan computer (Don Mills, Ontario).

PART 3 RESULTS

3.1 Histological Features of the Murine Placenta on Day 15

A brief illustrative summary of the histological features of the various cells of the 15 d murine placenta, the trophoblast cells in particular, is provided here before their labeling patterns are analyzed.

The labyrinthine trophoblast zone (Fig. 3) is primarily composed of labyrinthine trophoblast cells lining large maternal sinusoidal spaces. As shown in Figures 4 and 5, these cells are large, usually uninucleate, occasionally binucleate, and their cytoplasm often protruding into the sinusoids. Isolated macrophage type cells are occasionally interposed between trophoblast cells and fetal capillaries which are usually abundant in this zone.

The spongiotrophoblast zone (Fig. 3) is a compact layer of spongiotrophoblast cells lining fewer and smaller sinusoidal spaces. As shown in Figures 4 and 5, these cells are larger, uninucleate and polygonal in shape. This zone, in some areas, extends as villous shaped projections into the labyrinthine zone and also makes contact with the maternal decidua. Fetal stromal cell clusters often remain within the core of the projections appearing in the labyrinthine layer. In certain planes of section, these villous or tongue-shaped projections may appear as islands of stromal cells with a surrounding mantle of spongiotrophoblast cells located within the labyrinthine zone (Figures 4 and 5). Tentative identification of these stromal cell clusters as being fetally derived in origin is justified later in this thesis.

FIGURE 4

Semithin sections through various regions of the 15-day old murine placenta. Sections were stained with Iron hematoxylin

(a) Low magnification of the various areas in the placenta. Note the small fetal stromal cell cluster situated within the labyrinthine zone (arrow).

D - Decidual cell area

LTZ - Labyrinthine Trophoblast Zone

STZ - Spongiotrophoblast Zone

(magnification x 189)

(b) Low magnification of a cluster of fetal stromal cells (arrows) situated within a sheath of spongiotrophoblast cells extending into the labyrinthine zone.

ST - Spongiotrophoblast cells

(magnification x 189)

COLOURED PICTURES
Images en couleur

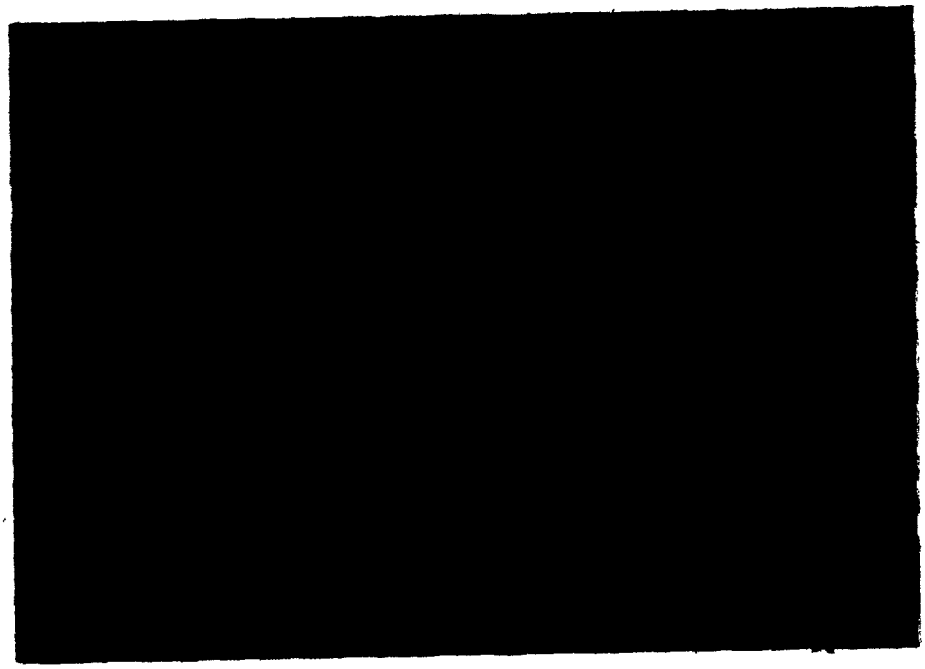


FIGURE 5

Semithin (0.5 μm thick) Epon sections through different regions of 15 day-old murine placenta. Sections were stained with Iron-hematoxylin.

(a) Labyrinthine zone.

C - Fetal capillary lumen
E - Fetal endothelial cell
LT - Labyrinthine Trophoblast cell
S - Maternal sinusoid
(magnification x 1000)

(b) Spongiotrophoblast zone

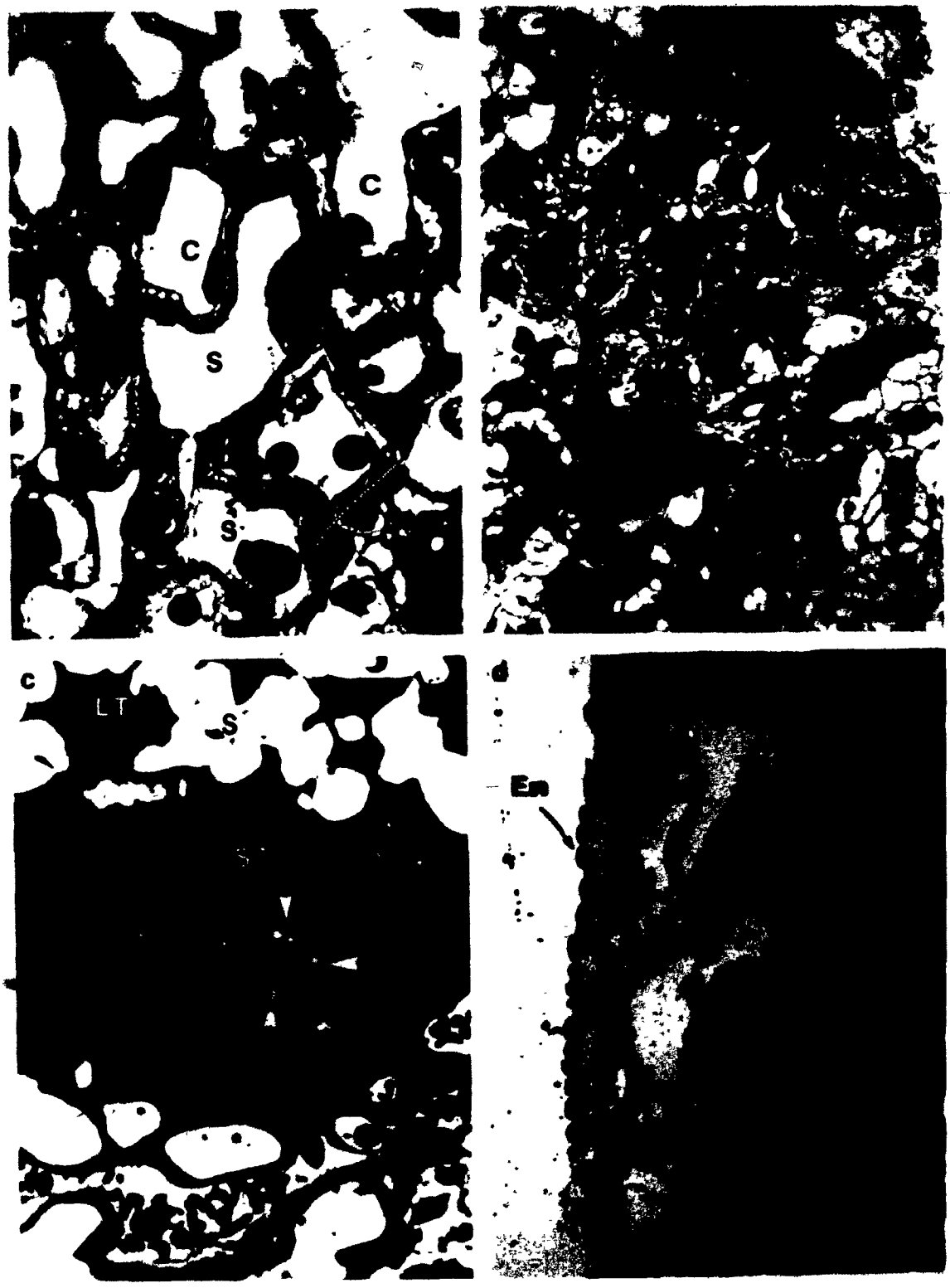
ST - Spongiotrophoblast cells
D - Decidual cells
(magnification x 400)

(c) Radioautograph showing fetally derived stromal cells located within a mantle of spongiotrophoblast cells. These cells (arrowheads) are characterized by containing a highly vacuolated cytoplasm and convoluted nucleus.

C - Fetal capillary lumen
LT - Labyrinthine trophoblast cell
ST - Spongiotrophoblast cell
S - Maternal sinusoid
(magnification x 480)

(d) Radioautograph of a section through the lateral region of the placenta

En - Yolk Sac Entodermal Cell
RM - Reichert's Membrane
(magnification x 400)


C
C
S
S
C
LT
S
Em

Trophoblast giant cells (Figures 3 and 6) are interposed as an incomplete layer between the spongiotrophoblast cells and the decidua, and embed into the decidua. These cells are extremely large, and may appear multinucleate.

Maternally derived decidual tissue is the key maternal component of the fetomaternal interface. This tissue consists primarily of typical stromal type "decidual cells" and infiltrating leucocytes. Decidual cells in semithin sections following the use of fixatives as described in the materials and methods, show nuclei sitting in cytoplasmic boundaries which appear essentially as empty spaces due to the loss of lipids which abound in these cells (Figures 4, 5 and 6).

On the fetal side of the placenta lies the entodermally derived fetal yolk sac which is composed of two layers: parietal and visceral. The parietal layer with the intervening Reichert's membrane (a thick layer of basal lamina) abutts on to the labyrinthine trophoblast cells. Yolk sac endodermal cells are cuboidal in shape with round nuclei (Figures 5 and 6). Occasionally free macrophages may be seen within the yolk sac cavity.

3.2 Radioactivity in the Blood

Radioactivity in the orbital venous blood samples were determined at various intervals after a single intravenous injection of I^{125}-labelled monoclonal anti H-$2K^k$ antibody in mice at 15 d of syngeneic and allogeneic pregnancy. Results were expressed as percent injected radioactivity/ml of blood (Fig. 7).

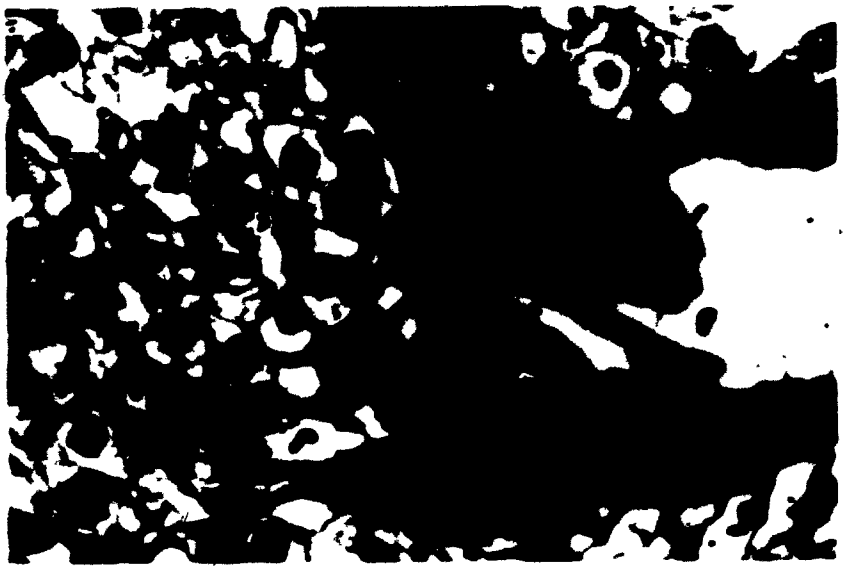
Assuming a total blood volume of approximately 2 ml in these mice

FIGURE 6

Semithin Epon section through the lateral regions of 15 day gestation murine placenta.

(a) D - Decidual cells
G - Giant Trophoblast cells
(magnification X 400)

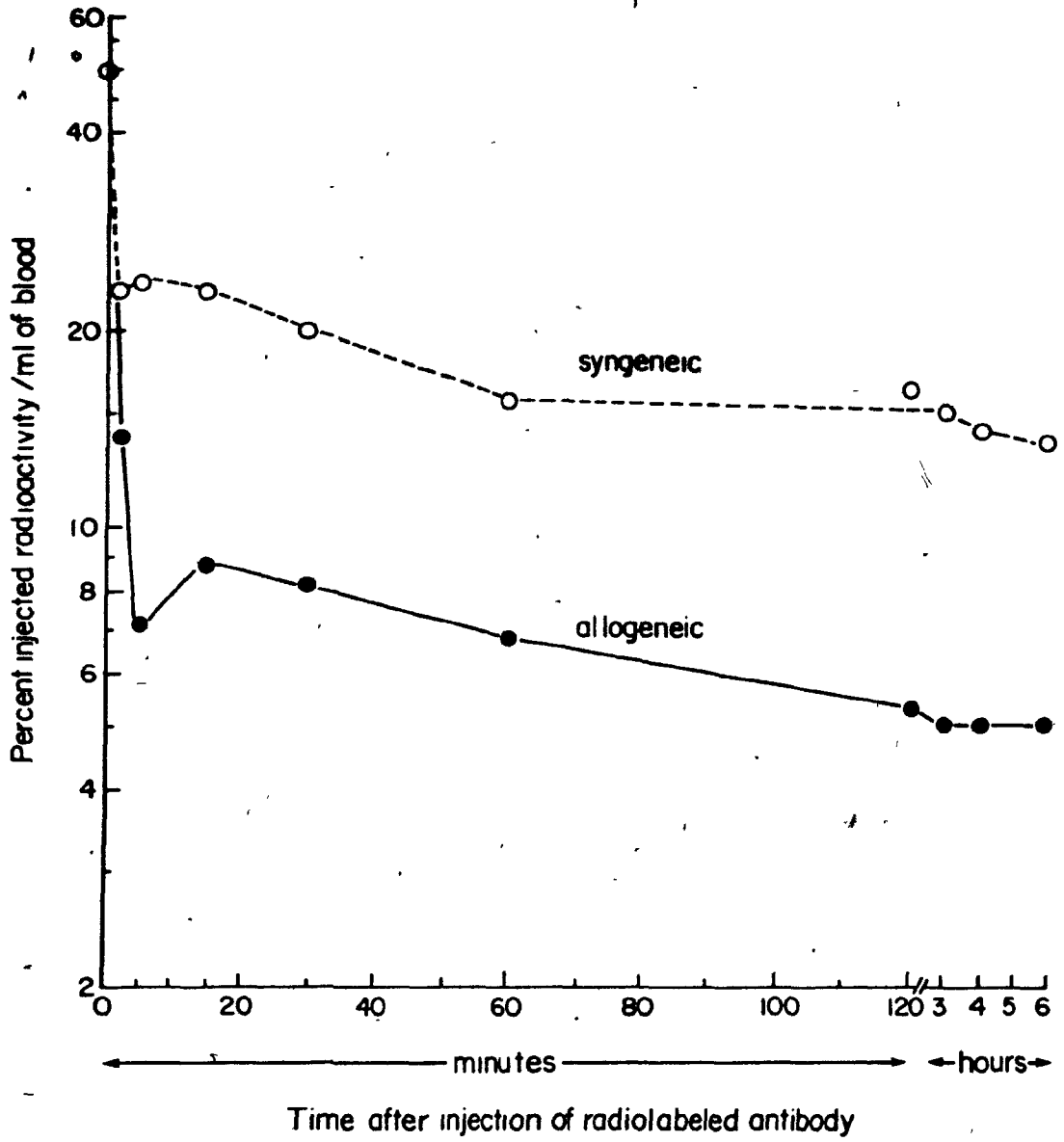
(b) En - Yolk sac Entodermal cells
RM - Reichert's Membrane
(magnification X 1000)



b

FIGURE 7

Blood clearance of I^{125}-anti-H-$2K^k$ antibody as a function of time in 15-day pregnant females. Each time point is an average of two animals.


60
40
20
10
8
6
4
2
Percent injected radioactivity /ml of blood
syngeneic
allogeneic
0
20
40
60
80
100
120
3
4
5
6
minutes
hours
Time after injection of radiolabeled antibody

at day 15 of gestation (8% of 25 gm body weight), the theoretical extrapolated zero time value in the blood/ml should be 50%.

In allogeneically pregnant mice, the clearance of radioactivity was very rapid during the first 5 minutes (T 1/2 of about 1.7 minutes) followed by a small rise between 5 and 15 min. The activity then declined more slowly (T 1/2 = 1.5 hr) up to 1 hour, following which the decline was extremely slow.

In syngeneically pregnant animals, the initial clearance was at a similar rate up to 2 minutes, followed by a minor rise between 2 and 5 minutes. The second slow phase of the decline, in this case, was between 5 min. and 1 hour, occurring approximately at the same rate as noted for allogeneic pregnancy. Following this period, the decline was, again, extremely slow. Retention of radioactivity in the blood in these animals from 5 minutes onwards was 2.5-3 times that in allogeneically pregnant mice. This finding can be explained by a higher extent of removal of the H-$2K^k$ ab by allogeneic placenta from the maternal circulation because of H-2 related binding by cells.

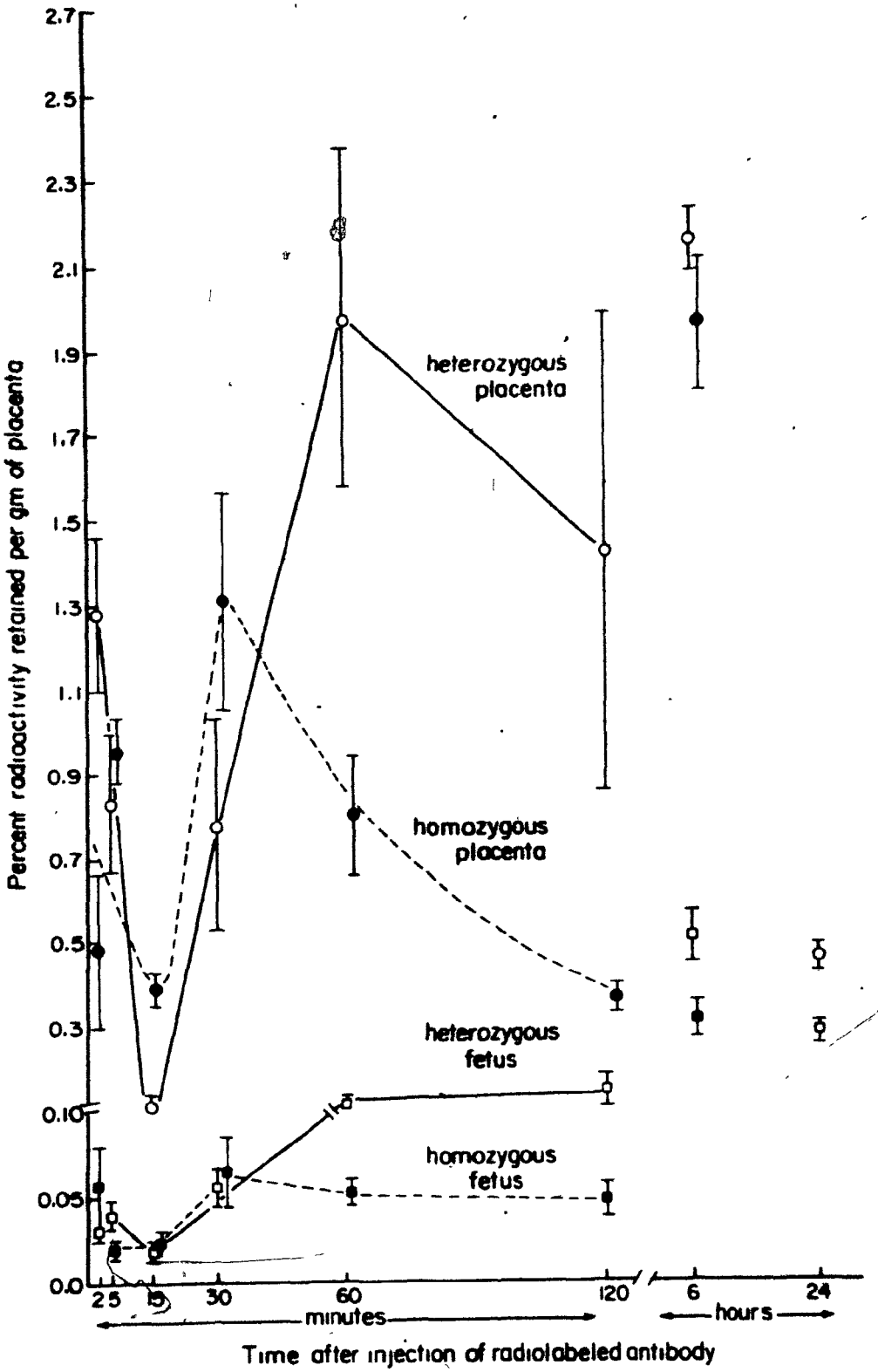
The precise reason for the small secondary rise or plateau of blood radioactivity (between 2 and 15 minutes) remains unknown. This may be due to a secondary release of radioactivity by some organs such as the liver which is known to process proteins including antibodies.

3.3 Radioactivity in the Placenta and the Fetus

Total radioactivity in the freshly isolated homozygous as well as heterozygous placenta and fetus expressed as percent retained per gram of wet tissue at different intervals is presented in Figure 8. There

FIGURE 8

A comparison of the percent retention of I^{125} -anti-H-$2K^k$ antibody at various time points by homozygous and heterozygous placentae and fetuses after a single injection of the monoclonal antibody. Each point represents the mean of 12 to 36 placentae and fetuses obtained from 2-6 animals. Vertical bars represent standard errors of the mean.


Percent radioactivity retained per gm of placenta
2.7
2.5
2.3
2.1
1.9
1.7
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.10
0.05
0.0
heterozygous placenta
homozygous placenta
heterozygous fetus
homozygous fetus
2.5
5
15
30
60
120
6
24
minutes
hours
Time after injection of radiolabeled antibody

was appreciable retention of radioactivity as early as 2 minutes in both placental types, the retention in the heterozygous type being significantly higher ($p < 0.05$) than in the homozygous type. Between 2 and 15 minutes there was a decline in the radioactivity in both cases. The activity then rose to a peak at 30 min. in homozygous placenta and 60 minutes in heterozygous placenta followed by a secondary decline up to 2 hours in both cases. Activity in the heterozygous form was significantly higher than in the homozygous form at all intervals from 60 minutes onwards ($p < 0.05$). This difference may be indicative of H-2 specific labeling of cells within the heterozygous placenta.

The initial high retention followed by a decline in radioactivity (2-15 min.) in both placental types is possibly due to some blood contamination of these organs in spite of the perfusion procedure. This explanation is based on some degree of similarity of their initial labeling pattern with the initial blood clearance curve (Figure 7). True retention because of cellular binding in the placenta is presented later from a radioautographic analysis of sections of placenta.

Both placental types reveal some secondary increase in activity between 2 and 6 hours, to a greater extent in the homozygous placenta. Twenty-four hour measurement done only in the heterozygous placenta shows a decline again. The reasons for the secondary rise observed at 6 hours is presently unknown.

Both types of fetuses revealed a very small retention of radioactivity compared to the respective placentae at all time intervals, the differences between the two types being insignificant

at earlier intervals (up to 30 min.) but significant ($p < 0.05$) at later intervals (60-120 min) when the activity in the heterozygous fetuses was higher.

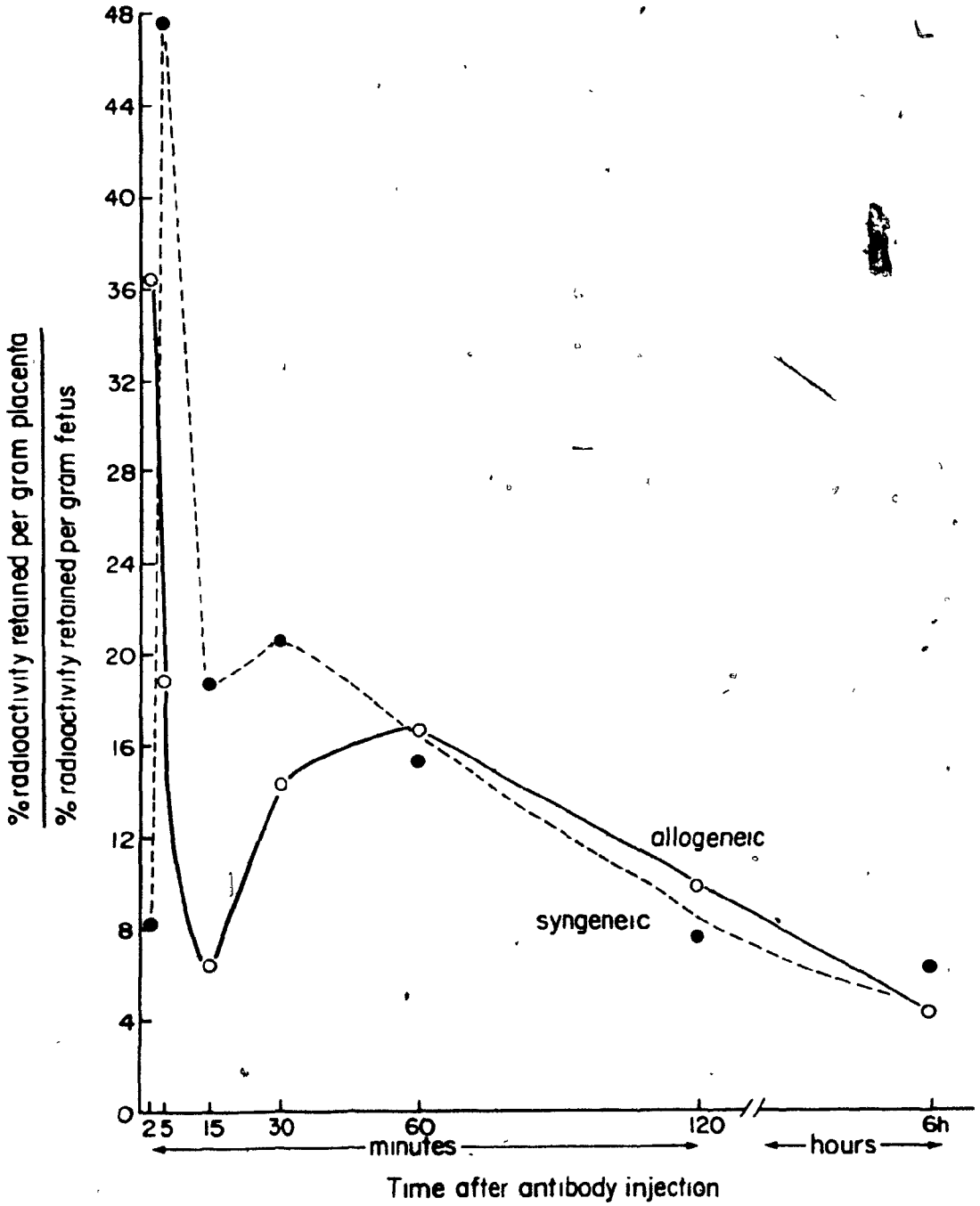
For the passage of antibody molecules to the fetus. they must cross the placental barrier from the maternal to the fetal circulation within the placenta. For this reason, the ratio of activity/gm of placenta to that of the fetus was plotted as a function of time in both types of pregnancies, to examine whether the heterozygous placenta was acting as a selective barrier to the passage of antibody molecules directed against the fetal H-2 antigens. The results are provided in Figure 9. Excepting at very early time points (2-5 min.), the ratios were not significantly higher in the heterozygous than in homozygous form ($p=0.45$). From 60 min. onwards, the values were nearly identical. These results suggest that although the heterozygous placenta retained a higher amount of labelled antibody, it did not act as a selective barrier to the passage of antibody molecules to the fetus compared to the homozygous placenta. It must be mentioned, however, that both placental forms acted as potent and equally efficient barriers to the passage of antibody molecules to the fetus (Figure 8).

3.4 Light Microscopic Labeling Pattern of Cells Within the Murine Placenta

The labeling pattern of various cell types within the heterozygous as well as homozygous placentae following the intravenous injection of radiolabeled monoclonal anti-$H2K^k$ antibody was examined using light microscope radioautography. Experiments were carried out

FIGURE 9

The mean ratio of the percent radioactivity retained per gram placenta to that of the fetus as a function of time in allogeneic and syngeneic pregnancies. The values are derived from the raw data in Figure 8.


% radioactivity retained per gram placenta
% radioactivity retained per gram fetus
48
44
40
36
32
28
24
20
16
12
8
4
0
allogeneic
syngeneic
2.5
15
30
60
120
6h
minutes
hours
Time after antibody injection

on day 15 of gestation only.

For the purpose of selecting the threshold number of silver grains for a positive labeling of cells within a placenta, the mean number of grains per unit area of 76 μm^2 of the lumen of fetal capillaries was assumed to represent the background in both the heterozygous (bxk) and homozygous (bxb) placentae. This background was chosen because of two reasons: (1) fetal capillaries were ubiquitous in all the trophoblast zones, and (2) their lumen should not bear any specific labeling. The mean grain density of the fetal capillary lumen (pooled from all regions of the placenta) at various time intervals is presented in Figure 10. The labeling patterns for both placental types are very similar. In both cases, the mean grain density shows some rise from 2 min. onwards reaching a peak at 30 min. and then showing a continuing decline up to the 2 hour interval.

3.4.1 The Labeling Pattern of Labyrinthine Trophoblast Cells

Figure 11 represents the labeling pattern of the labyrinthine trophoblast cells as given by the mean number of silver grains per unit area at various time points. In both placental types the labeling reaches a peak at 60 min. declining at 2 hr. Homozygous trophoblast labeling is only marginally above background between 2 min. to 60 min., indicative of a small degree of non H-2 related (possibly Fc receptor related) antibody binding. At 2 hrs the value was indistinguishable from the background. Heterozygous trophoblast labeling was significantly ($p<0.05$) above the background (fetal capillary lumen) at all time points. The values were also consistently higher than those

FIGURE 10

A measurement of the number of grains per unit area ($76\mu m^2$) of the lumen of fetal capillaries during allogeneic and syngeneic pregnancies at different time intervals after the injection of the radiolabeled anti-H-$2K^k$ antibody. Each point represents the mean grain density of 3 placentae obtained from 3 pregnant animals. Vertical bars represent standard errors of the mean.

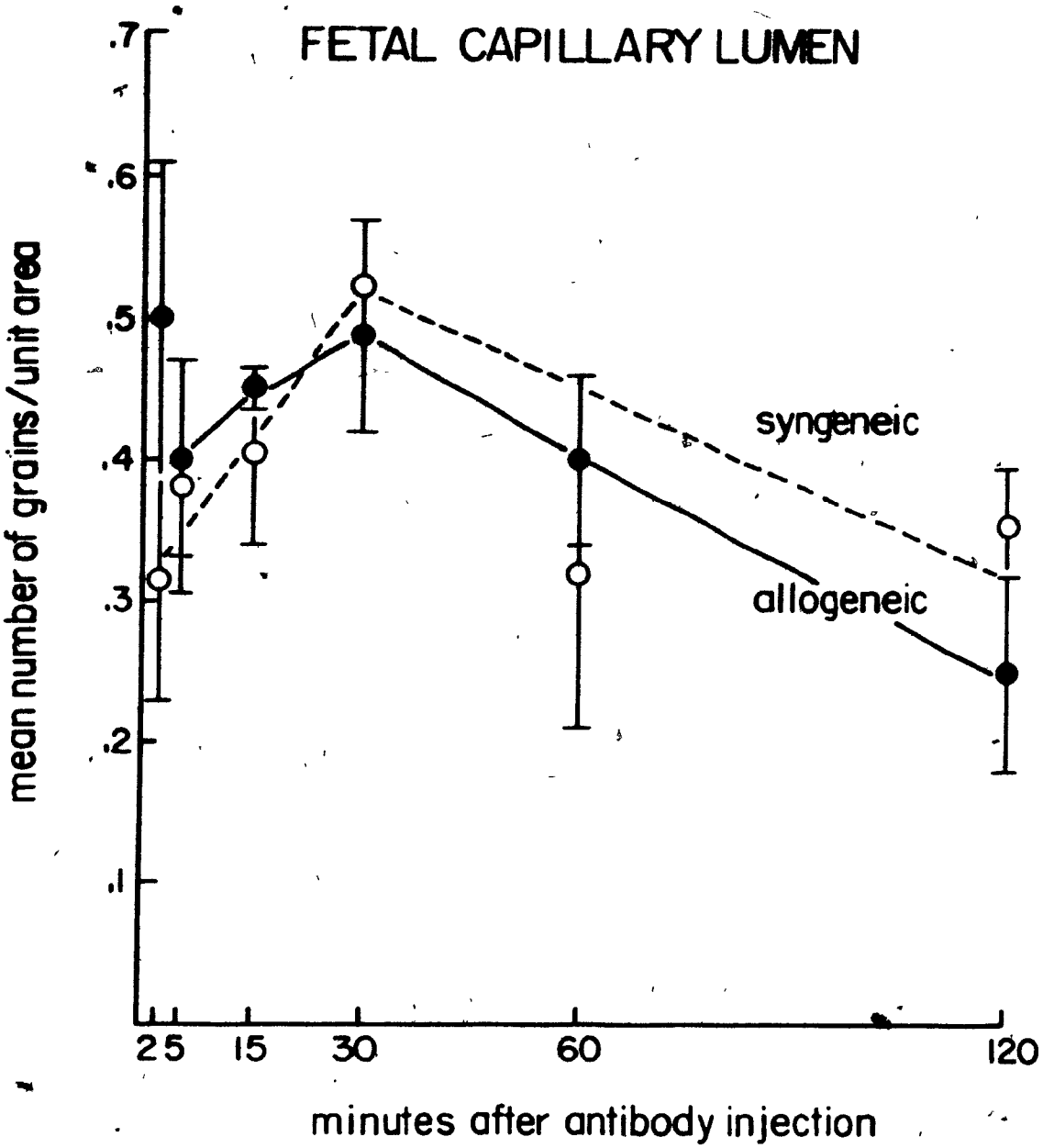

FETAL CAPILLARY LUMEN
mean number of grains/unit area
.7
.6
.5
.4
.3
.2
.1
syngeneic
allogeneic
2 5
15
30
60
120
minutes after antibody injection

FIGURE 11

The labeling pattern of the labyrinthine trophoblast cells derived from 15 day heterozygous (allogeneic pregnancy) and homozygous (syngeneic pregnancy) placentae.

Each point represents the mean grain density observed on two to three placentae obtained from 2 to 3 animals. Vertical bars represent the standard errors of the mean.

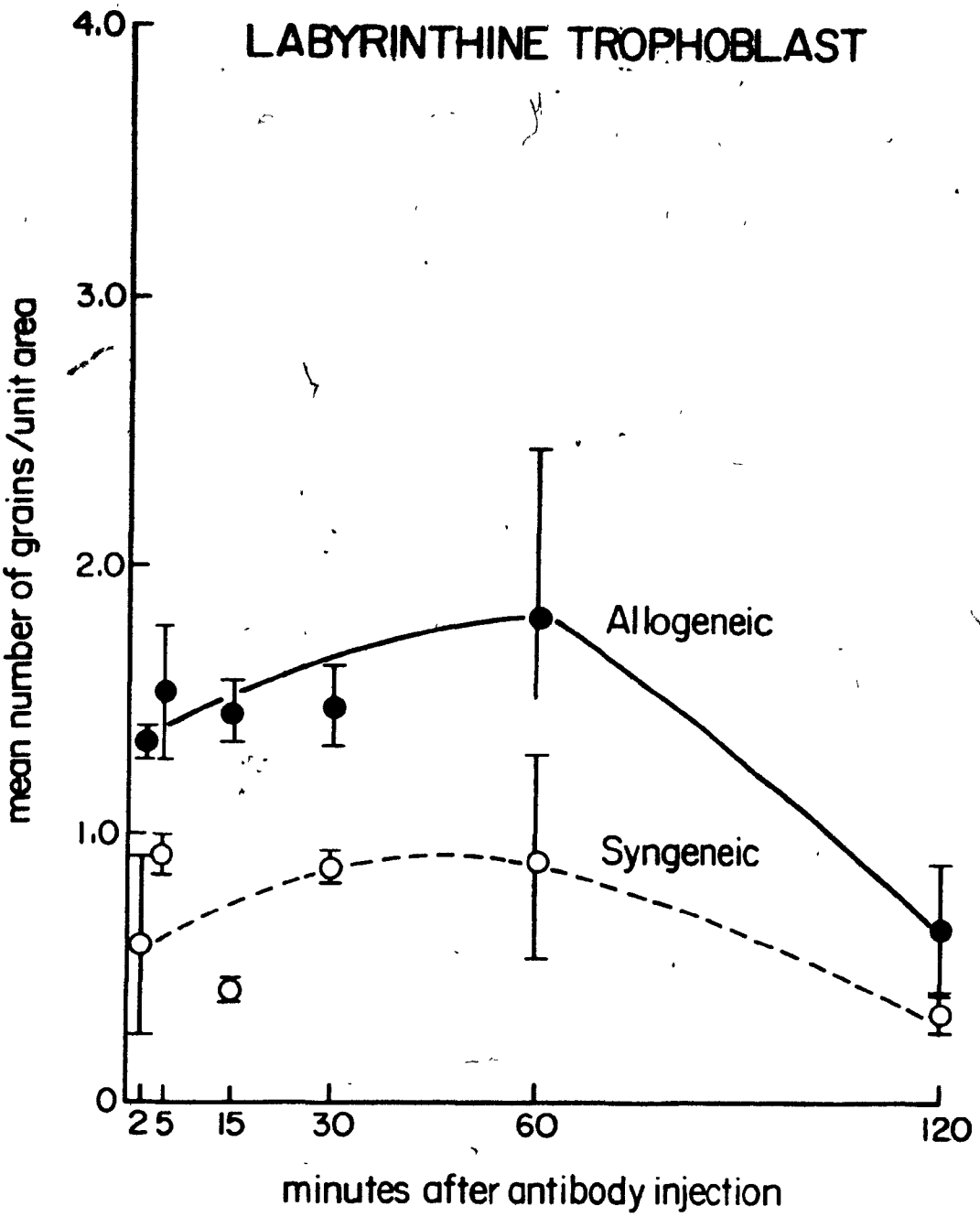

LABYRINTHINE TROPHOBLAST
mean number of grains/unit area
4.0
3.0
2.0
1.0
0
Allogeneic
Syngeneic
2 5
15
30
60
120
minutes after antibody injection

FIGURE 12

Light microscope radioautographs of the labyrinthine region of the 15d placentae at 2 to 30 min after the injection of I^{125}-anti-H-$2K^k$ antibody. Sections are 0.5 μm thick and stained with Iron hematoxylin

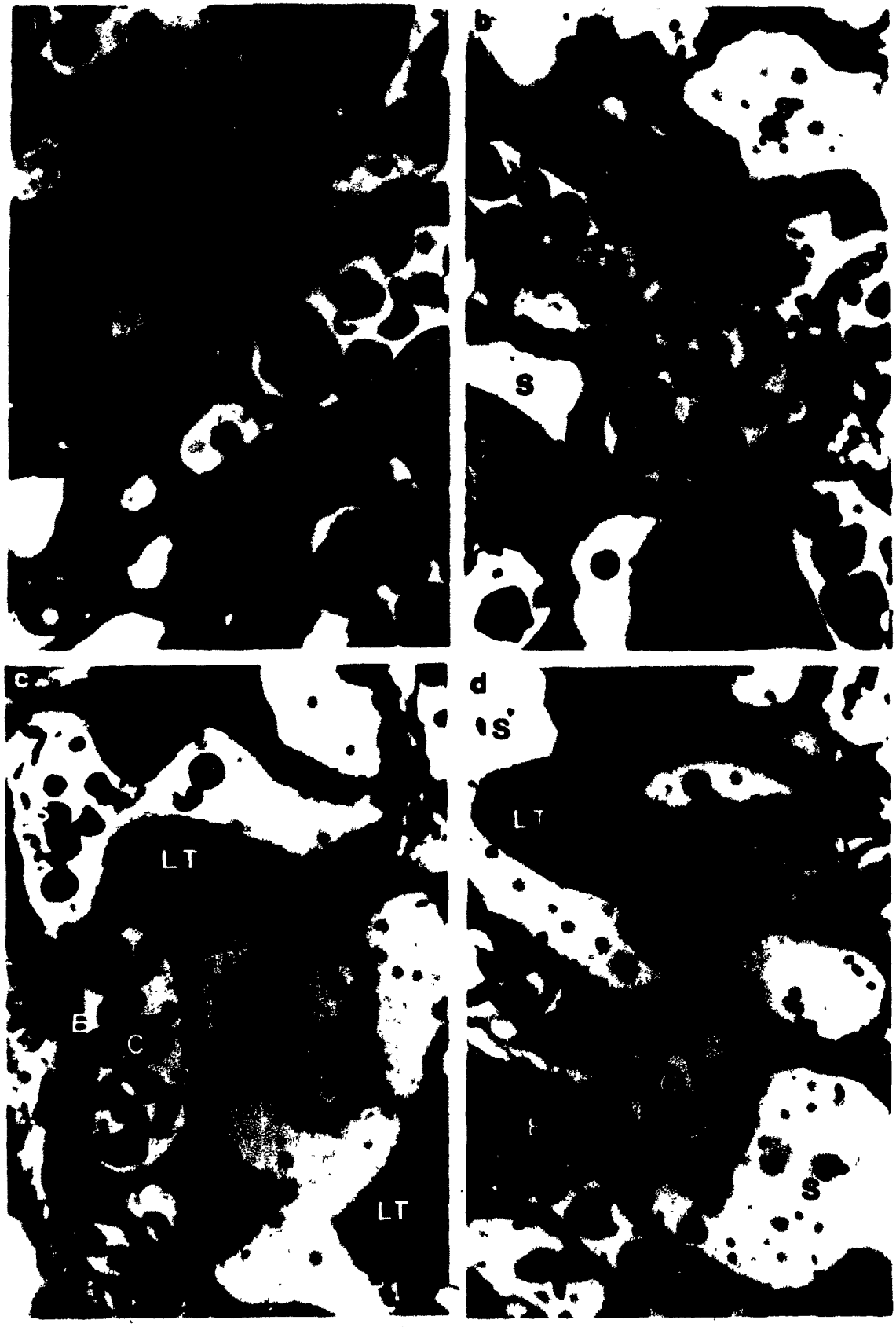
a) Homozygous, 2 min
b) Heterozygous, 2 min
c) Homozygous, 30 min
d) Heterozygous, 30 min

Note specific labeling of heterozygous labyrinthine trophoblast cells increasing with time. Very weak labeling observed on homozygous labyrinthine cells at 30 min is non-H-2 related, explicable on the basis of Fc receptors.

Note specific labeling of heterozygous, fetal capillary endothelial cells at 2 min and to a greater extent at 30 min. All magnifications are 1200x.

Legend:

LT - Labyrinthine Trophoblast Cell
C - Fetal Capillary lumen
S - Maternal sinusoid
E - Endothelial cell


b
S
c
LT
B
C
LT
d
S
LT
S

of homozygous trophoblast cells at all time points, eg. three fold higher at 2 min. to 60 minutes. The differences were significant at $p = 0.1$ at 2 min. to 30 minutes. The differences in labeling intensity of the heterozygous over homozygous trophoblast reflected specific H-2 related antibody binding, indicating the presence of H-2 antigens on these cells. Radioautographs showing labeling of labyrinthine trophoblast cells are illustrated in figure 12.

3.4.2 The Labeling Pattern of Spongiotrophoblast Cells

Positive labeling of spongiotrophoblast cells of the heterozygous placenta above background occurs only at 30 min. and 60 min. after the injection of the antibody (figure 13). The mean grain density rises to 3 fold that of homozygous trophoblast cells at these time points, followed by a decline at 2 hrs to a value indistinguishable from that of the homozygous trophoblast cells. The peak value of 1.12 ± 0.015 grains/area is attained at 30 minutes, which is significantly higher ($p < 0.005$) than the homozygous placenta. At no time point was homozygous spongiotrophoblast labeling significantly higher than the background except at 5 min where the labeling was only marginally higher ($p=0.30$) indicating some non H-2 related antibody binding at this time point. A possible explanation for specific labeling of heterozygous spongiotrophoblast cells at time intervals later than seen for labyrinthine trophoblast cells may result from a slower accessibility of the antibody to these cells because of sparcity of maternal sinusoids in this zone.

FIGURE 13

Labeling pattern of spongiotrophoblast cells within the placentae derived from allogeneic and syngeneic pregnancies. Each point represents the mean of 2 to 3 placentae obtained from 2 to 3 animals. Vertical bars indicate standard errors of the mean.

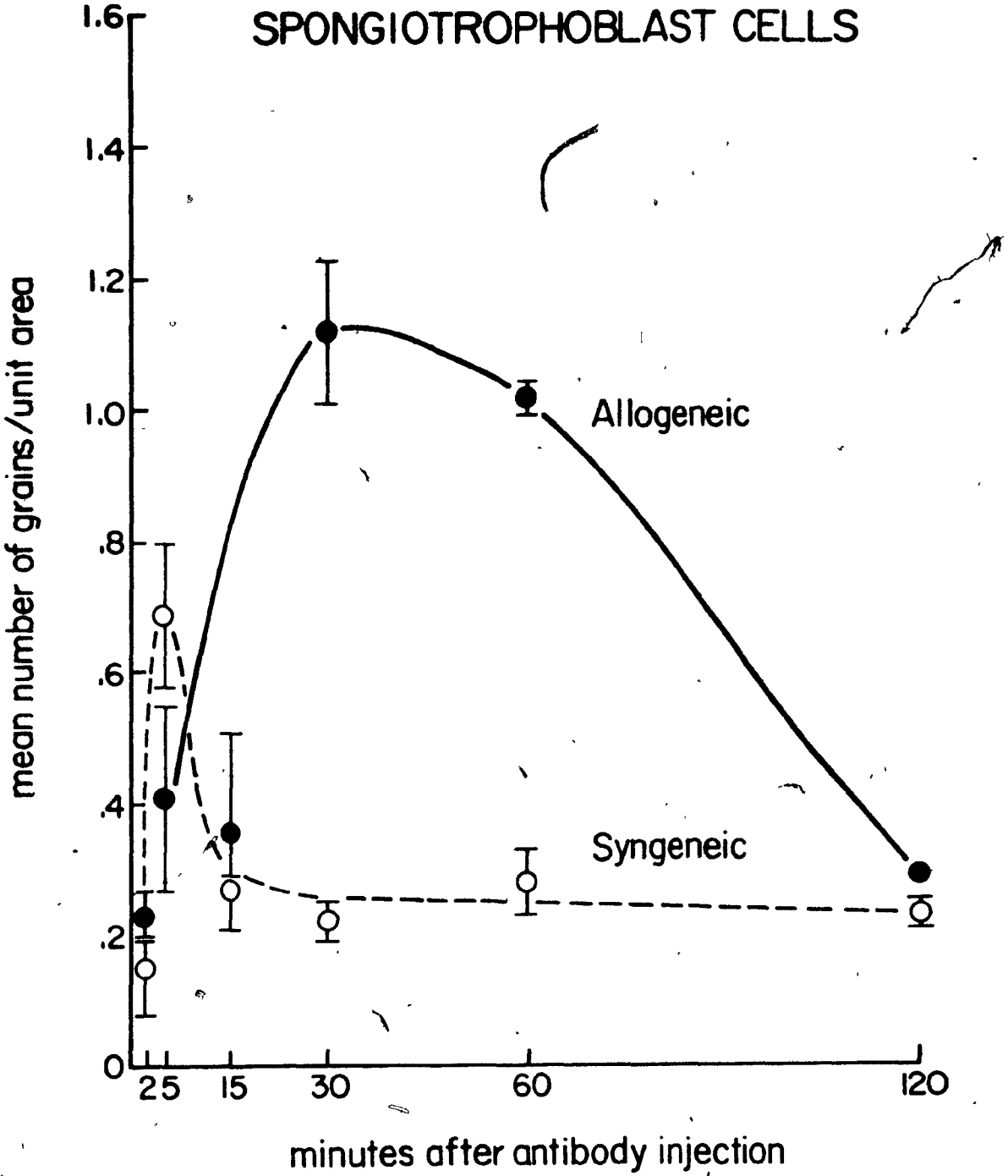

SPONGIOTROPHOBLAST CELLS
mean number of grains/unit area
1.6
1.4
1.2
1.0
.8
.6
.4
.2
0
Allogeneic
Syngeneic
2.5
15
30
60
120
minutes after antibody injection

FIGURE 14

Radioautographs of sections through the spongiotrophoblast region of placentae at various time periods after the injection of I^{125}-anti-H-$2K^k$ antibody.

a) Homozygous 15 min, showing no labeling above background (magnification x 1200)

b) Heterozygous 15 min, showing marginal labeling (magnification x 800)

c) Homozygous 30 min, showing marginal labeling (magnification x 1200)

d) Heterozygous 30 min, showing moderate labeling (magnification x 1200)

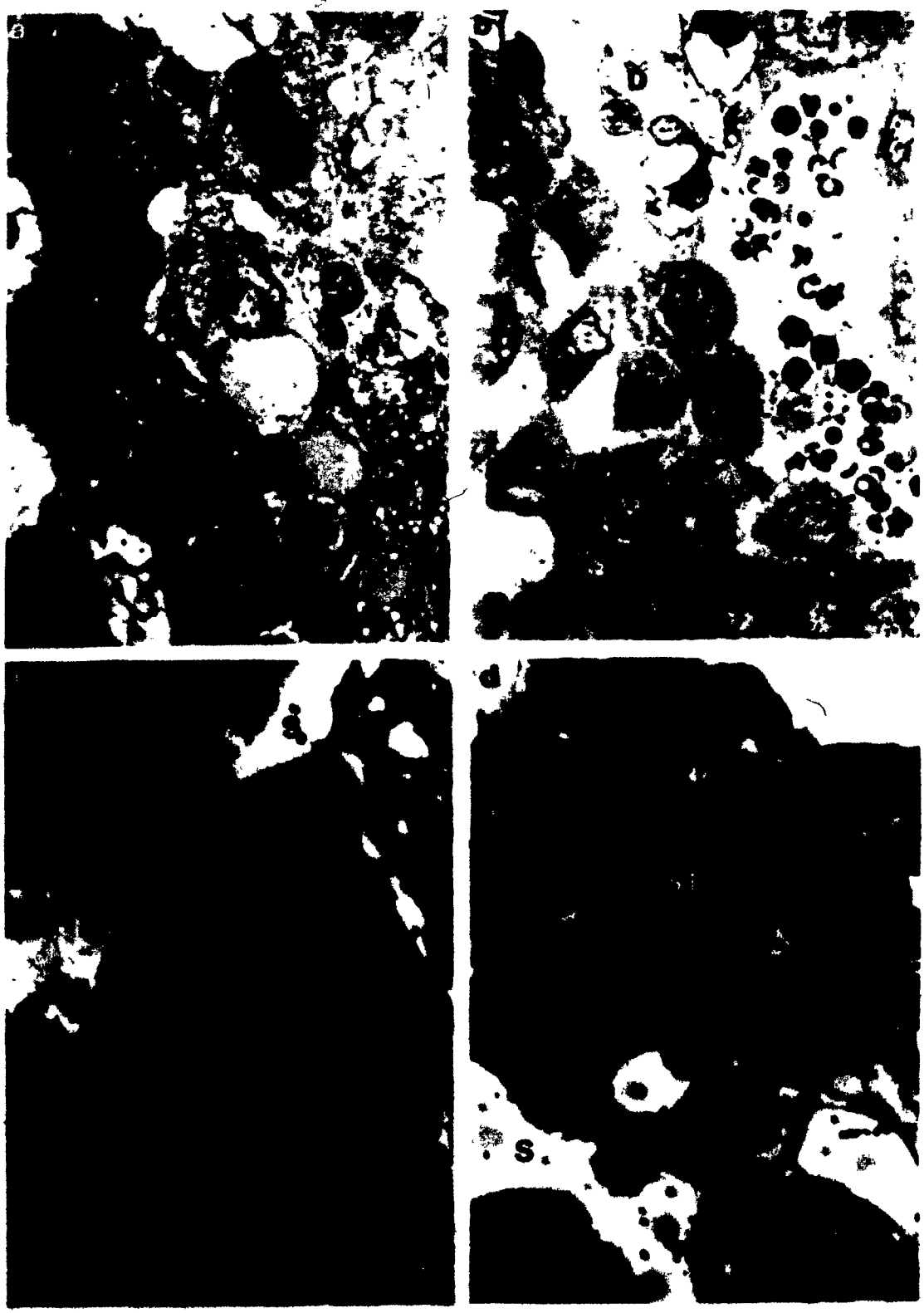
Decidual cells in (b) don't show any labeling above background.

C - Fetal capillary lumen

D - Decidual cell

S - Maternal sinusoid

ST - Spongiotrophoblast cell


a
b
S

From these results it may be concluded that spongiotrophoblast cells do express H-2 antigens. Labeling of these cells is illustrated in Figure 14.

3.4.3 The Labeling Pattern of Fetal Stromal Cell Clusters

Figure 15 demonstrates temporal labeling patterns for the clusters of fetally derived stromal cells seen within the heterozygous and homozygous placentae. Radioautographic preparations are presented in Figures 16, 18 and 19. These clusters are mostly located within the labyrinthine zone and to some extent in the spongiotrophoblast zone. In the former zone they are usually surrounded by a mantle of spongiotrophoblast cells. These stromal cells are 14-20 μm in diameter and are characterized by having convoluted nuclei, highly vacuolated and granular cytoplasm; features are somewhat similar to those described for "Hofbauer cells" in the human placenta (Boyd and Hamilton, 1970). Our nomenclature of "stromal cell clusters" has been assigned to them in the absence of a better terminology.

The stromal cell labeling patterns are qualitatively similar for both placental types (Figure 15) but their labeling intensities are different. The labeling intensity rises to a peak at 60 min and then declines at 2 hrs. However, the mean grain density of the stromal cell clusters in the heterozygous placenta is 1.5 to 4 times greater than the homozygous placenta, with a difference significant at $p < 0.05$ at 30 min. and $p=0.25$ at all other time intervals.

The stromal cell clusters in the heterozygous placenta show significant labeling above background ($p < 0.005$) at all time points.

FIGURE 15

Labeling pattern of fetal stromal cell clusters at various time periods after the intravenous injection of I^{125}-anti-H-$2K^{k}$ antibody into 15 day gestation pregnant female mice from allogeneic and syngeneic matings.

Each point represents the mean grain density of 2 to 3 placentae obtained from 2 to 3 animals. Vertical bars represent the standard error of the mean.

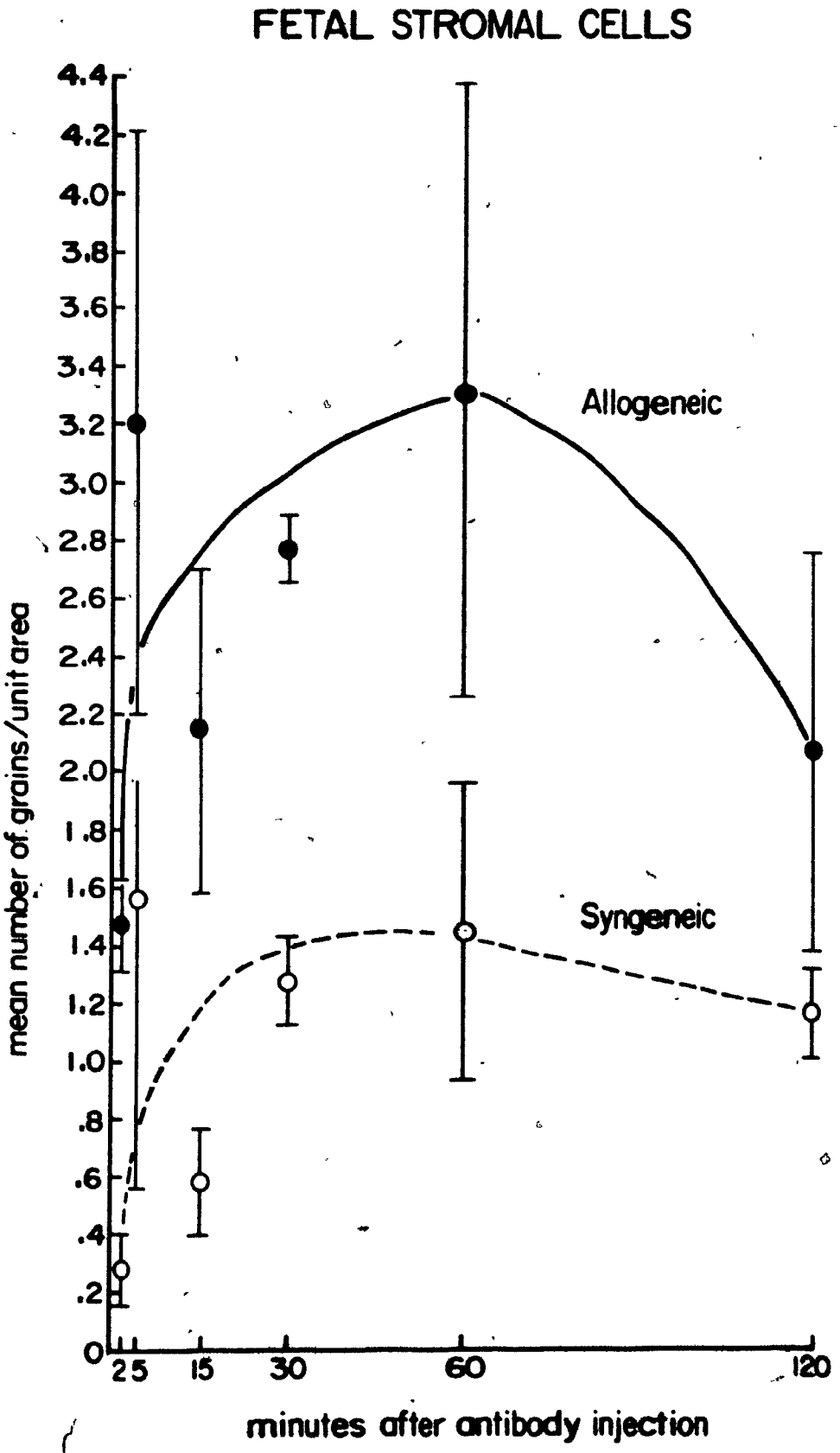

FETAL STROMAL CELLS
4.4
4.2
4.0
3.8
3.6
3.4
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
0
mean number of grains/unit area
Allogeneic
Syngeneic
25
15
30
60
120
minutes after antibody injection

FIGURE 16

Radioautographs of sections through the labyrinthine region of placentae at 1 hr after the injection of I^{125}-anti-$H2K^k$ antibody.

a) Homozygous: Labyrinthine zone, showing weak labeling

b) Heterozygous Labyrinthine zone, showing strong labeling

c) and d) show fetal stromal cell clusters (arrows and arrowheads) within a mantle of spongiotrophoblast cells extending into the labyrinthine region.

C Homozygous, showing little or no labeling

D Heterozygous, showing strong labeling

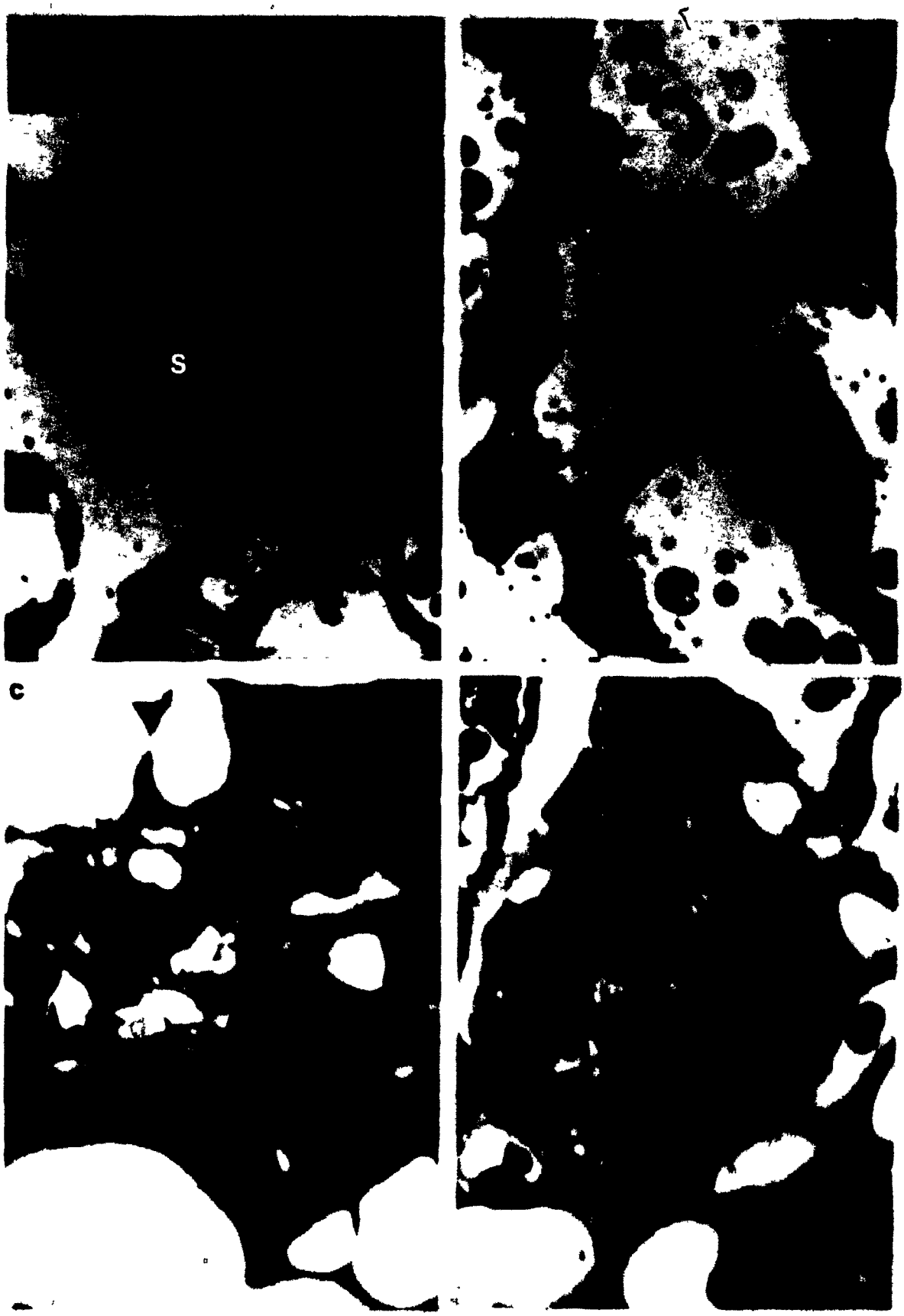
All magnifications are x 1200

C - Fetal capillary lumen

LT - Labyrinthine Trophoblast Cell

ST - Spongiotrophoblast cell

S - Maternal sinusoid


S
c

The homozygous placenta reveals significant labeling above background at $p<0.05$ at all time points except 2 min. and 15 min. after the injection of antibody.

These results indicate some non H-2 related antibody binding by homozygous stromal cell clusters possibly via Fc receptors. Much stronger labeling of the heterozygous cells is attributed to (a) the presence of a high concentration of H-2 antigens on their cell surface, (b) binding of antigen-antibody complexes via Fc receptors or both. These possibilities will be discussed further later.

3.4.4 Labeling Pattern of the Endothelium of Fetal Capillaries

Figure 17 represents the labeling pattern of the endothelium of fetal capillaries from heterozygous and homozygous placentae. Both placentae demonstrated labeling above background at all time points, the labeling of the heterozygous placenta being always higher than the homozygous placenta except at the 2 hr interval. The peak labeling occurs between 30 min. (homozygous placenta) and 60 min. (heterozygous placenta). The labeling intensity was very strong for the heterozygous placental cells (e.g. 3.49 ± 0.015 grains) at 60 minutes. The values were significantly higher ($p<0.05$) than in those for the homozygous cells at 2 min., 30 min. and 60 min. At 15 min. and 2 hrs the differences in the mean grain densities are significant at $p=0.25$.

Radioautographic labeling of fetal endothelium is illustrated in Figure 18.

These results indicate (a) a rapid transport of antibody molecules or their products to the fetal capillary endothelium (b) some binding

FIGURE 17

Labeling pattern of the endothelium of fetal capillaries of placentae, obtained from allogeneic and syngeneic pregnancies, observed at different time intervals after the injection of the radiolabeled anti-H-$2K^k$ antibody.

Each point represents the mean grain density of 2 to 3 placentae. Vertical bars represent the standard error of the mean.

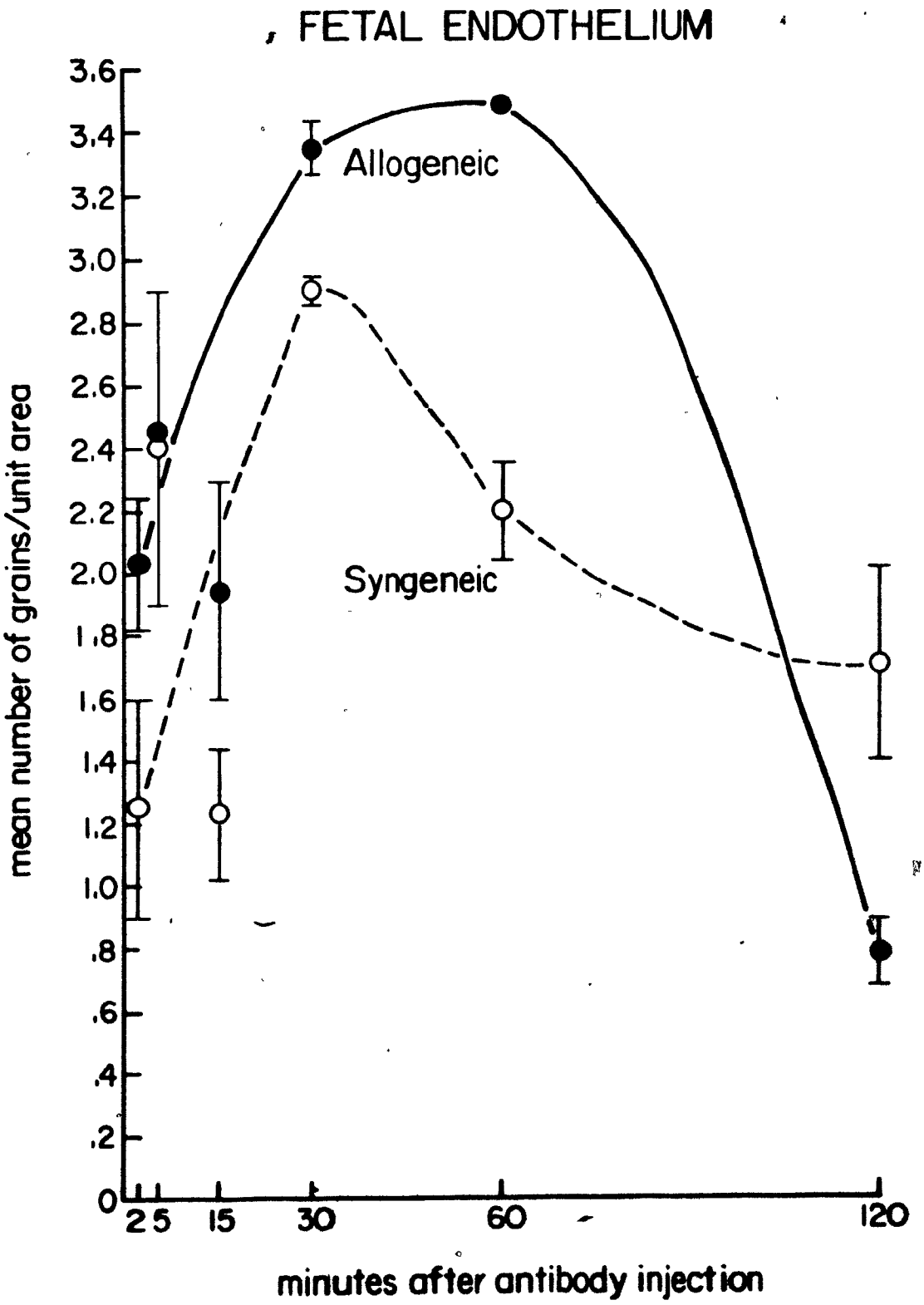

FETAL ENDOTHELIUM
Allogeneic
Syngeneic
mean number of grains/unit area
3.6
3.4
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
0
2.5
15
30
60
120
minutes after antibody injection

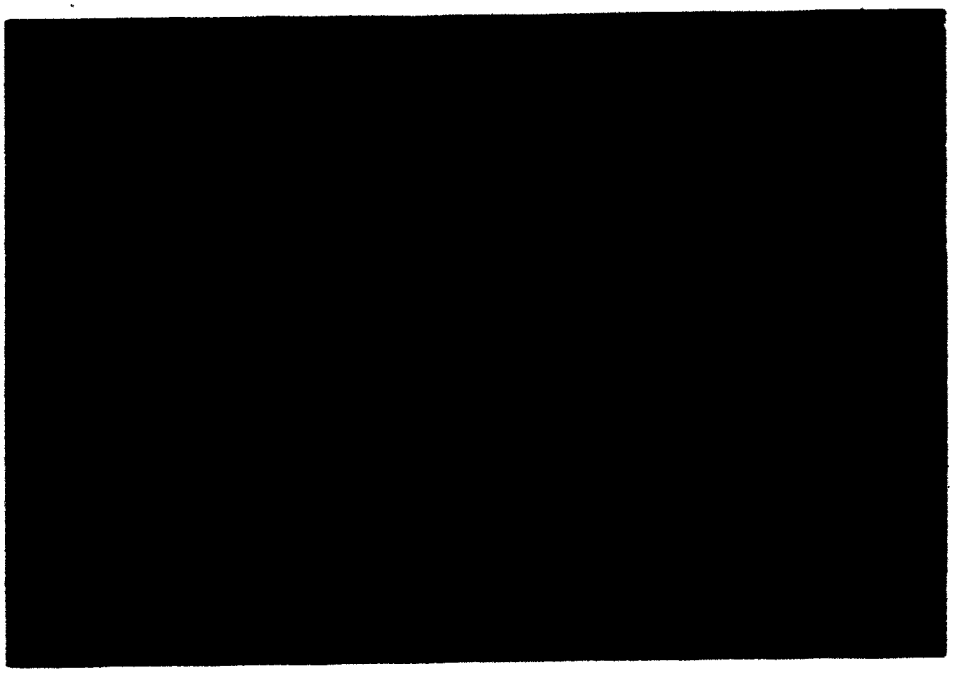
FIGURE 18

Radioautographs of sections through the labyrinthine zone of heterozygous (C57 BL x CBA) F_1, placentae after an intravenous perfusion of I^{125}-H-$2K^k$ antibodies.

a) Labeling of fetal endothelial cells (E) at 15 min.
(magnification x 1200)

b) Heavy labeling of fetally derived stromal cell cluster at 1 hr. Note silver grains on vacuolated cells.
(magnification x 1200)

COLOURED PICTURES
Images en couleur



of these molecules via non H-2 related mechanisms possibly via Fc receptors allowing for the labeling of homozygous cells and (c) additional H-2 related labeling of heterozygous endothelial cells either due to the presence of H-2 antigens or binding of antigen-antibody complexes or both.

3.4.5 Labeling Pattern of Macrophages

Mean grain densities of isolated macrophages located occasionally in the maternal sinusoids, in the fetal stroma of the placenta or within the yolk sac cavity (figures 19 and 20) show good labeling above background ($p<0.05$) in both the heterozygous and homozygous placentae (Table II). The labeling intensity of the macrophages from the heterozygous placenta was 4 time higher than that of the homozygous placenta at 2 min. and was approximately 3 times higher at later intervals.

As concluded earlier for fetal stromal cell clusters as well as fetal endothelial cells, macrophage labeling in homozygous placentae is explicable by Fc receptor mediated binding of antibody. Additional H-2 related labeling of heterozygous macrophages can, again, be explained by the presence of H-2 antigens, or binding of antigen- antibody complexes or both.

3.4.6 Labeling Pattern of Other Cells and Structures

The Reichert's membrane from the heterozygous and homozygous placenta, represented in Table II demonstrated labeling above background at all time intervals, indicating non specific binding of the radio-

FIGURE 19

Radioautographs of sections through different regions of heterozygous placentae at 30 min. to 1 hr. after the intravenous injection of I^{125}-H-$2K^k$ antibody.

a) At 30 min., showing a strongly labeled fetal stromal cell cluster (arrows) within a spongiotrophoblast core extending into the labyrinthine zone. (magnification x 800)

b) At 15 min., labyrinthine zone: A heavy labeled isolated macrophage. (magnification x 1200)

c) At 1 hr, yolk sac region: Strongly labeled macrophages within the yolk sac cavity (arrows) and some weak non specific labeling of the Reichert's membrane. (magnification x 800)

d) At 30 min, trophoblast giant cell layer bordering the maternal decidua. Neither cell type shows labeling. (magnification x 1200)

C - Fetal capillary lumen

D - Decidual cell

G - Giant Cell

En- Yolk Sac Entoderm

M - Macrophage

RM - Reichert's membrane

S - Maternal Sinusoid

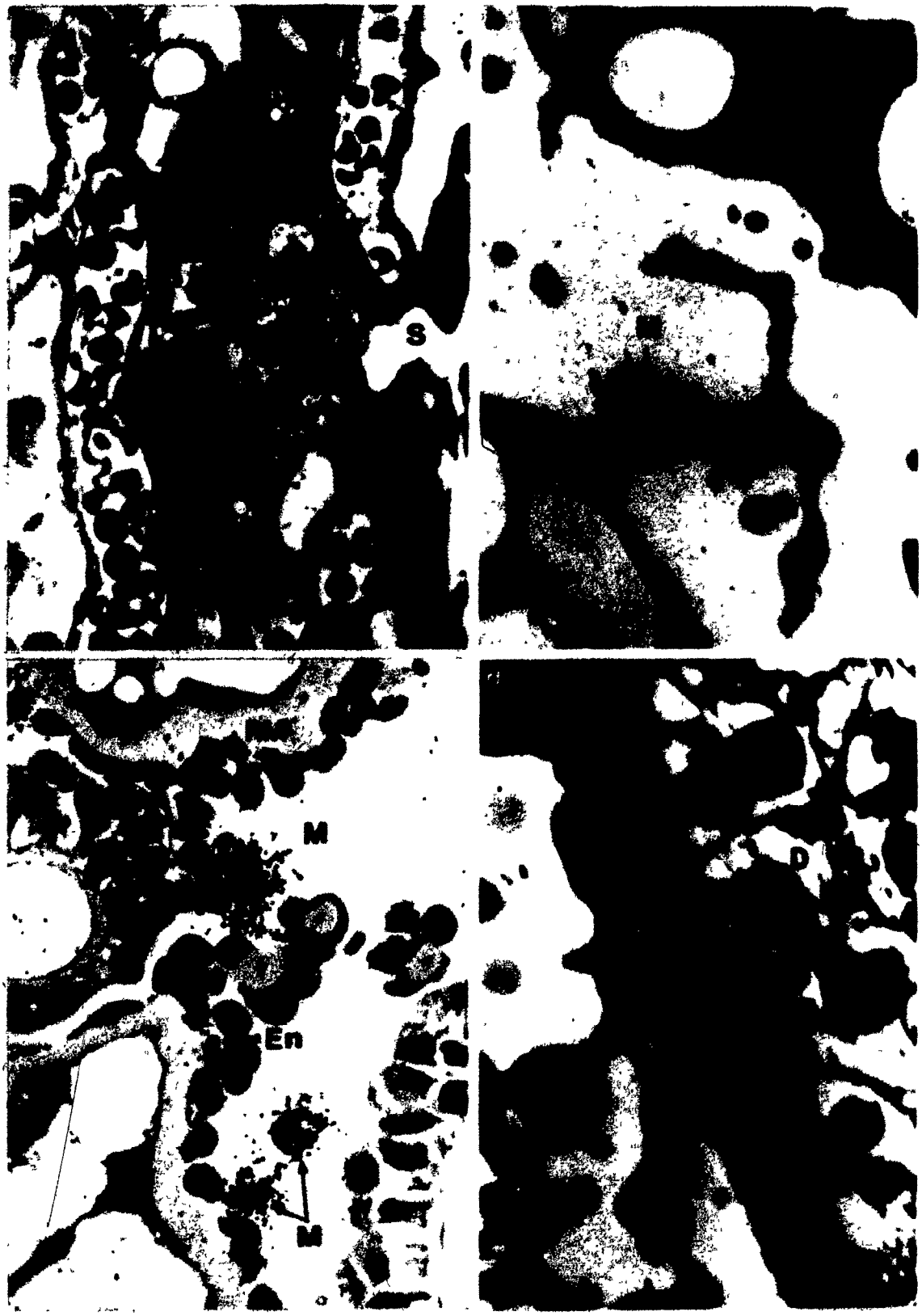

S
M
En
M

FIGURE 20

Radioautographic preparations of sections through the murine heterozygous placenta at different time points following the injection of I^{125}-anti-H-$2K^k$ antibody.

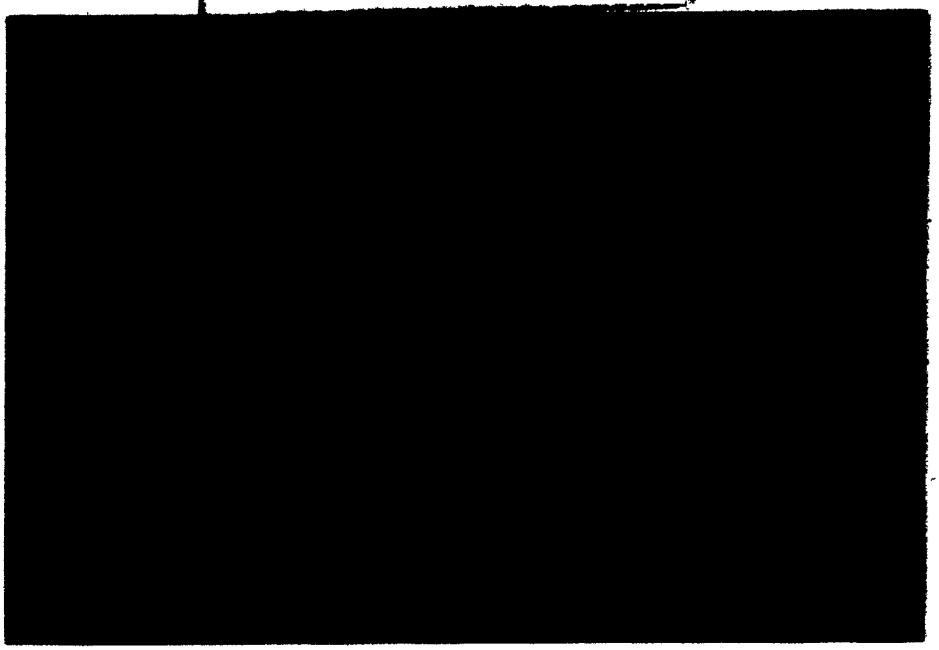
a) At 2 min., a strongly labeled isolated, identifiable macrophage (M) is observed in the labyrinthine trophoblast zone.

(magnification x 1200)

b) At 30 min., a macrophage (arrow) located within the yolk sac cavity demonstrates heavy labeling. Yolk sac entodermal cells (En) show very little labeling while Reichert's membrane (RM) shows good labeling.

(magnification x 1200)

COLOURED PICTURES
Images en couleur



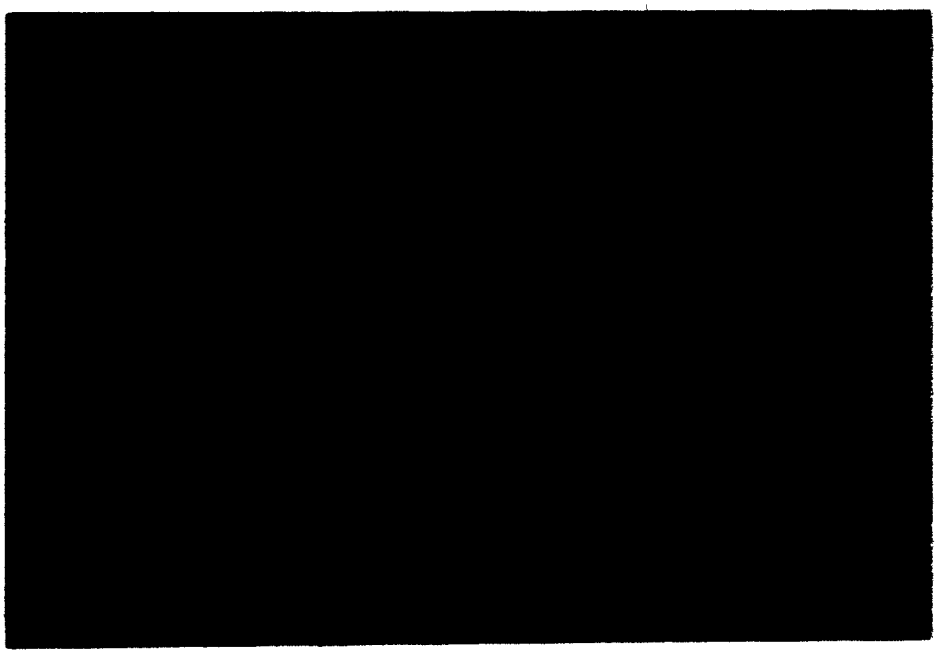


TABLE II

Grain Counts (mean ± standard deviation per unit area of various components of the placenta)

Structure	2 minutes		5 min.		15 min.		30 min.		60 min.		120 min.	
	allo.	syng.	allo.	syng.	allo.	syng.	allo.	syng.	allo.	syng.	allo.	syng.
decidual cells	0.27	0.35	0.33+ .06	0.54	0.21+ .06	0.29	0.60+ .03	0.21	0.46+ 0.11	0.51+ .01	0.29+ .01	0.23+ .01
trophoblast giant cells	0.21+ .05	0.24+ .04	0.31	0.63	0.38+ .05	0.33+ .1	0.52+ .27	0.42+ .19	0.47+ .15	0.35+ .06	0.24+ .16	0.28+ .09
Reichert's membrane	2.74+ .62	4.19	nd	3.04+ .21	1.05+ .02	0.97+. .04	nd	1.3+.3	2.70+ 0.51	1.95+ .48	0.9+ 0.1	1.69+ .08
yolk sac	0.51+ .04	.54	nd	0.67+ .00	0.48+ .01	0.86+ .08	nd	0.49+ .15	1.24	0.43+ .10	0.87+ .07	0.56+ .09
macrophages	23.6+ 0.59	6.13+ .536	16.43+ 1.37	3.03+ 0.52	7.53+ 0.38	1.6+ 0.26	7.97+ 0.74	1.77+ 0.26	7.63+ 0.64	2.4+ 0.45	8.1+ 0.81	2.63+ 0.26

nd - not determined because of a paucity of the structures

Values without standard deviations were derived from single animal because of a paucity of structures in the sections from other animals.

labeled antibodies. Furthermore, there is a variation in the extent of labeling at different time intervals with the highest labeling seen at 2 minutes for the homozygous placenta and at 5 minutes for the heterozygous placenta. Labeling of the Reichert's membrane is illustrated in Figures 19 and 20.

As noted in Table II, labeling of decidual cells of the maternal uterus and most importantly the trophoblast giant cells (also illustrated in Fig. 19) was not significantly above background at any interval for both placental types.

Endodermal cells of the parietal and visceral yolk sac (Figure 19 and 20) did show some marginal labeling above background at most time points, the level of significance being small ($p > 0.2 < p\ 0.25$). This may be attributed to the presence of Fc receptors.

3.5 H-2 Related Versus Non H-2 Related Retention of Antibody by Placental Cells Under Different Conditions.

3.5.1 A Comparison of Fc Receptor Density in the Two Types of Placentae.

Light microscope radioautographic studies revealed some non H-2 related binding of the radiolabeled monoclonal anti $H2K^k$ antibody to cells within the placenta which do not possess the target antigens. This may, in part or in whole, be due to a binding to the Fc receptors on various cells within the placenta. To determine the extent of Fc related as opposed to H-2 related binding, a radiolabeled monoclonal anti-HLA-DR antibody was injected intravenously into mothers carrying either placental type, and the total radioactivity was measured per

gram of placenta. Table III presents the percent retained radioactivity at 2 minutes, when cellular binding is expected to be confined to the cell membrane. The results reveal that the binding in the heterozygous placenta is not higher, and in fact slightly lower than in the homozygous placenta. The binding of anti-HLA-DR antibody to the homozygous placenta is comparable to that of anti-H-$2K^k$ (Fig. 8).

Since the results of the above experiment may also be complicated to some extent by some unbound activity in the blood, if any, contaminating the perfused placentae, an in vitro binding assay with dispersed placental cells was also performed with anti-HLA-DR antibody. Results presented in Table IV shows essentially similar binding by cells of both placental types after 30 min. incubation at 4°C.

These results indicate the presence of an identical incidence of Fc binding sites (receptors) in cells of both placental types.

3.5.2 Affinity of Antibody Binding Via H-2 Antigens or Fc Receptors.

Anti-H-$2K^k$ antibody apparently bound to the heterozygous placental cells by two mechanisms (1) via H-2 antigenic sites and (2) via Fc receptors on the cell membrane. Binding to the homozygous placental cells was by the second mechanism above. To compare the antibody binding affinity by the two mechanisms an <u>in vitro</u> experiment was designed in which dispersed placental cells of both types were exposed to radiolabeled anti-H-$2K^k$ antibody in the presence of excess unlabeled antibody (at 1:10 ratio) at 4°C to examine the <u>effect</u> on total bound cpm to 20×10^6 cells (Table V). Again, heterozygous placental cells

TABLE III

Percent radioactivity retained per gram placenta at 2 min. after the injection of radiolabeled anti-HLA-DR[a] antibody in vivo.

Placenta Type	% Radioactivity Retained
heterozygous	0.20%
homozygous	0.326%

[a] 9.8×10^7 cpm in 1 µg of anti-HLA-DR Ab

TABLE IV

In vitro binding of radiolabeled anti-HLA-DR antibody [a] to dispersed placental cells

Placenta Type	Total Bound cpm
heterozygous	37 090
homozygous	38 490

a $3X10^7$ cpm in 1 µg of anti-HLA-DR antibody was incubated with $10X10^6$ cells at 4°C for 30 min.

TABLE V

The effects of excess nonradiolabeled antibody[b] on the binding of radiolabeled antibody[a] to placental cells at 4°C

Placenta Type	Cell Number	Incubated With	Total Bound cpm
heterozygous	$20x10^6$	I^{125}-$\alpha H2K^k$ alone	58 523
		I^{125}-$\alpha H2K^k$+$\alpha H2K^k$	18 393
homozygous	$20x10^6$	I^{125}-$\alpha H2K^k$ alone	36 066
		I^{125}-$\alpha H2K^k$+$\alpha H2K^k$	21 657

a $1.9x10^7$ cpm in 1 µg of anti-H-$2K^k$ antibody

b 10 µg of anti-H-$2K^k$ antibody

NOTE: α = anti

demonstrated a greater labeling with the radiolabeled antibody alone, the difference being explained by H-2 related binding. Excess unlabeled antibody reduced the amount of bound radioactivity in the heterozygous placental cells to a greater degree (69%) than in the homozygous cells (40%). This finding indicates that there is a higher affinity for anti-H-2K^{k} antibody binding to the H-2 antigenic sites than to the Fc receptors on placental cells at 4°C, since the incidence of Fc receptors was identical for both placental types.

3.5.3 Effects of Temperature on H-2 Versus FcR Related Labeling.

Since all the *in vivo* labeling experiments were done at body temperature i.e. approx. 37°C, an *in vivo* binding experiment was designed to compare the retention of the anti-H-2K^{k} antibody by homozygous and heterozygous placental cells at 37°C, as opposed to 4°C. Results given in Table VI shows that the total cpm increased 1.7 fold in heterozygous cells as opposed to 1.6 fold in homozygous cells when temperature was changed from 4° to 37° under the labeling conditions provided. This experiment was repeated under slightly different labeling conditions indicated in Table VII. Under these conditions the labeling increased 1.3 fold with heterozygous placental cells as compared to 1.1 fold with homozygous cells. Thus a change from 4°C to 37° causes a slightly more effective antibody retention by the heterozygous placental cells. This may suggest that the shift of temperature (4°C to 37°C) improved the H-2 related labeling more efficiently than Fc-receptor related labeling. This hypothesis was tested from the results of binding displacement caused by excess non radioactive IgG at both temperatures in the two types of placental cells (Table VII). Excess nonradioactive IgG can only displace FcR

TABLE VI

In vitro radiolabeled monoclonal anti-H-$2K^k$ antibody[a] binding to dispersed placental cells at different temperatures

Placenta Type	Cell Number	Incubation Temperature	Total Bound cpm
heterozygous	$10x10^6$	4°C	37 104
		37°C	63 452
		(37°C/4°C labeling ratio = 1.7)	
homozygous	$10x10^6$	4°C	18 620
		37°C	30 284
		(37°C/4°C labeling ratio = 1.6)	

a Cells were incubated with 1 µg ($1.9x10^7$ cpm) of I^{125}-anti-H-$2K^k$ antibody for 30 min.

TABLE VII

In vitro radiolabeled monoclonal anti-H-2K^k antibody retention by dispersed placental cells under different conditions

Placenta Type	Cell No.	Incubated With	Incubation[3] Temp.	Total Bound cpm
heterozygous	$20x10^6$	(a) Ab alone[1]	4°C	36 908
		(b) Cold IgG +Ab[2]	4°C	14 039
		(c) Ab alone	37°C	49 065
		(d) Cold IgG+Ab	37°C	32 707
homozygous	$20x10^6$	(e) Ab alone	4°C	22 048
		(f) Cold IgG+Ab	4°C	6 556
		(g) Ab alone	37°C	24 247
		(h) Cold IgG+Ab	37°C	8 411

1 $2.07x10^7$ cpm in 1 µg of I^{125}-anti-H-2K^k

2 Cells were exposed to 188 µg nonradioactive mouse IgG for 30 min followed by radiolabeled anti-H-2K^k for 30 min.

3 Incubated for 30 min period

Ratios c/a = 1.3
g/e = 1.1
d/b = 2.3

a-e = 14860 cpm (approx H-2 related labeling)

For interpretations, please see text (Results)

related binding in both cell types. Assuming that FcR incidence was similar in both placental types (Table VII), the difference a-e should approximate H-2 related labeling in the heterozygous placenta, a value which is close to the value of (b) following a displacement of FcR related binding with excess nonradioactive IgG in the heterozygous placental cells. However, there may still remain a minor degree of FcR related binding as suggested by the residual counts (f) in the homozygous placenta. Nevertheless, raising the temperature from 4° to 37°C improved the labeling of IgG treated heterozygous placental cells 2.3 fold as given by the ratio d/b. This finding supports the hypothesis that the rise in the temperature improved the H-2 related labeling more efficiently than FcR related labeling. A higher labeling at 37°C in both cases may in part result from an internalization of receptor-ligand complexes.

3.6 Electron Microscopic Labeling Pattern of Cells Within the Murine Placenta

A qualitative analysis of the labeling pattern at the ultrastructural level was performed for various cell types in the heterozygous and the homozygous placentae at various time intervals. The degree of labeling at the light microscope level was often used as a guide in the selection of the materials for a finer analysis.

3.6.1 Labyrinthine Trophoblast

At 2 minutes after the injection of the radiolabeled antibody, significant preferential labeling was localized on the sinusoid lining plasma membrane of the heterozygous labyrinthine trophoblast cells

(Figures 21 and 22), but little or no labeling of these sites was noted on the homozygous labyrinthine trophoblast cells lacking the target antigens. These results, for the first time, have conclusively demonstrated the presence of paternal type H-2 antigens on the sinusoidal face of the plasma membrane of the labyrinthine trophoblast. Furthermore, plasma membrane associated labeling was also observed in the middle trophoblast layer of the trichorial labyrinth at 2 minutes (Figure 21a) demonstrating that these cells also bear the paternal haplotype class 1 MHC antigens.

At 5 minutes following the injection of the antibody (Figure 23), the labeling pattern was similar to that seen at 2 minutes, but, in addition, labeling was seen on the plasma membrane of the monocytoid cells present in the interstices of the labyrinth. This labeling may be due to H-2 antigenic sites located on these cells (putatively fetal in origin) or due to a binding of antigen-antibody complexes (transferred from trophoblast cells) via their Fc receptors.

Furthermore, at this time point, movement of label was observed within the cytoplasm of the trophoblast as well as monocyte-macrophage type cells. The labeling was associated with membrane bound vesicles, including coated vesicles and lysosomes. These findings suggest a transport of label from the plasma membrane to the intracellular sites where the process of degradation as well as membrane recycling may occur.

At 15 minutes following the injection, label appears on the plasma membrane of the deepest (3rd) trophoblast layer of the trichorial labyrinth in the heterozygous placenta (Figure 24). Thus, all the three layers of the labyrinthine trophoblast cells express

FIGURE 21

Electron microscope radioautograph of the labyrinthine region of the heterozygous placenta at 2 min. after the injection of the radiolabeled anti-H-2K^k antibody.

(a) The trichorial nature of the labyrinthine trophoblast is shown as three layers numbered 1, 2 and 3. Label is seen on the microvilli of the sinusoidal plasma membrane (arrows). Some label is also seen on the plasma membrane of cells in the layer 2 (mag. x 2000).

(b) Magnified region of part of (a) indicated by arrows showing the label on the microvilli (x 10,000).

(c) Shows sinusoidal plasma membrane labeling as in (a) but from a different placenta. Some silver grains can also be seen on fragments of microvilli within the sinusoidal lumen (arrows) (x 2000)

(d) Similar labeling at a higher magnification (x 4000) from another area of the same placenta as in (c)

S = Maternal Sinusoid

C = Fetal Capillary

LT = Labyrinthine Trophoblast Cell

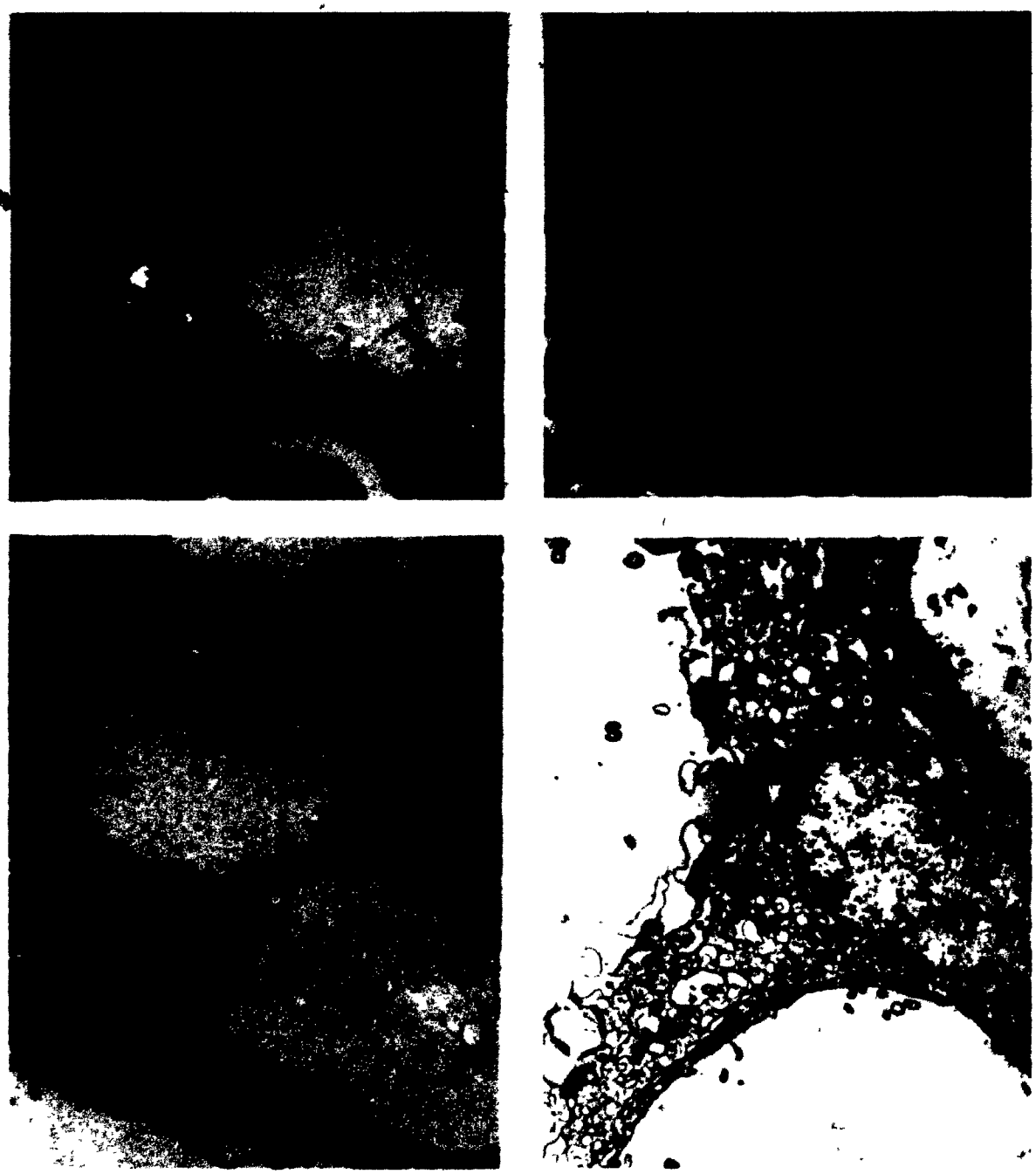


FIGURE 22

Labyrinthine region of a heterozygous placenta at 2 min. to show H-2 related labeling of plasma membrane of labyrinthine trophoblast cells

(a) Silver grain shown on a microvillus (Mv) (x 27,000)

(b) Labeling on sinusoidal plasma membrane including microvilli seen in cross section (x 14,000)

(c) Labeling on plasma membrane of trophoblast cytoplasmic extensions in cross sections (1 and 2) lying adjacent to a monocytoid cell (Mo) (x 6700)

(d) A higher magnification of area 1 in (c) (x 27,000).

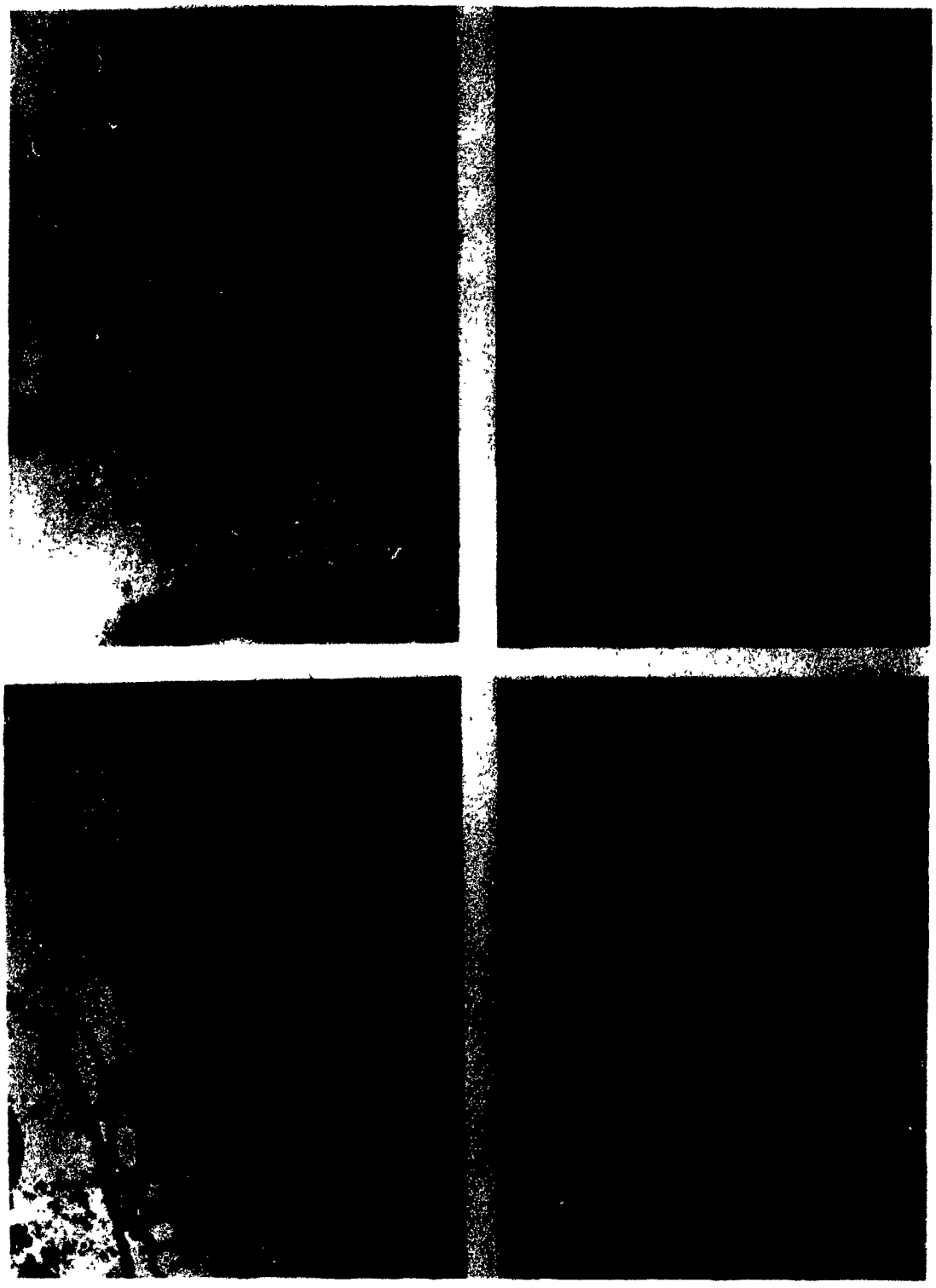


FIGURE 23

Labyrinthine region of the heterozygous placenta at 5 min.

(a) Three layers of trichorial labyrinth are shown as 1, 2, 3. Silver grains are seen on the plasma membrane of cell layer 2 (x 5,000).

(b) A monocyte-macrophage type cell in the interstices of the labyrinth shows both plasma membrane bound labeling (arrows) as well as intracellular labeling (arrowheads) (x 4,000).

(c) A higher magnification of the intracellular silver grains in (b). Grains are associated with coated vesicles (arrows) and a lysosome (arrowhead) (x 14,000)

(d) Shows silver grains associated with a membrane bound vesicle close to the plasma membrane (arrows) in a sinusoid lining the labyrinthine trophoblast (x 5,000).

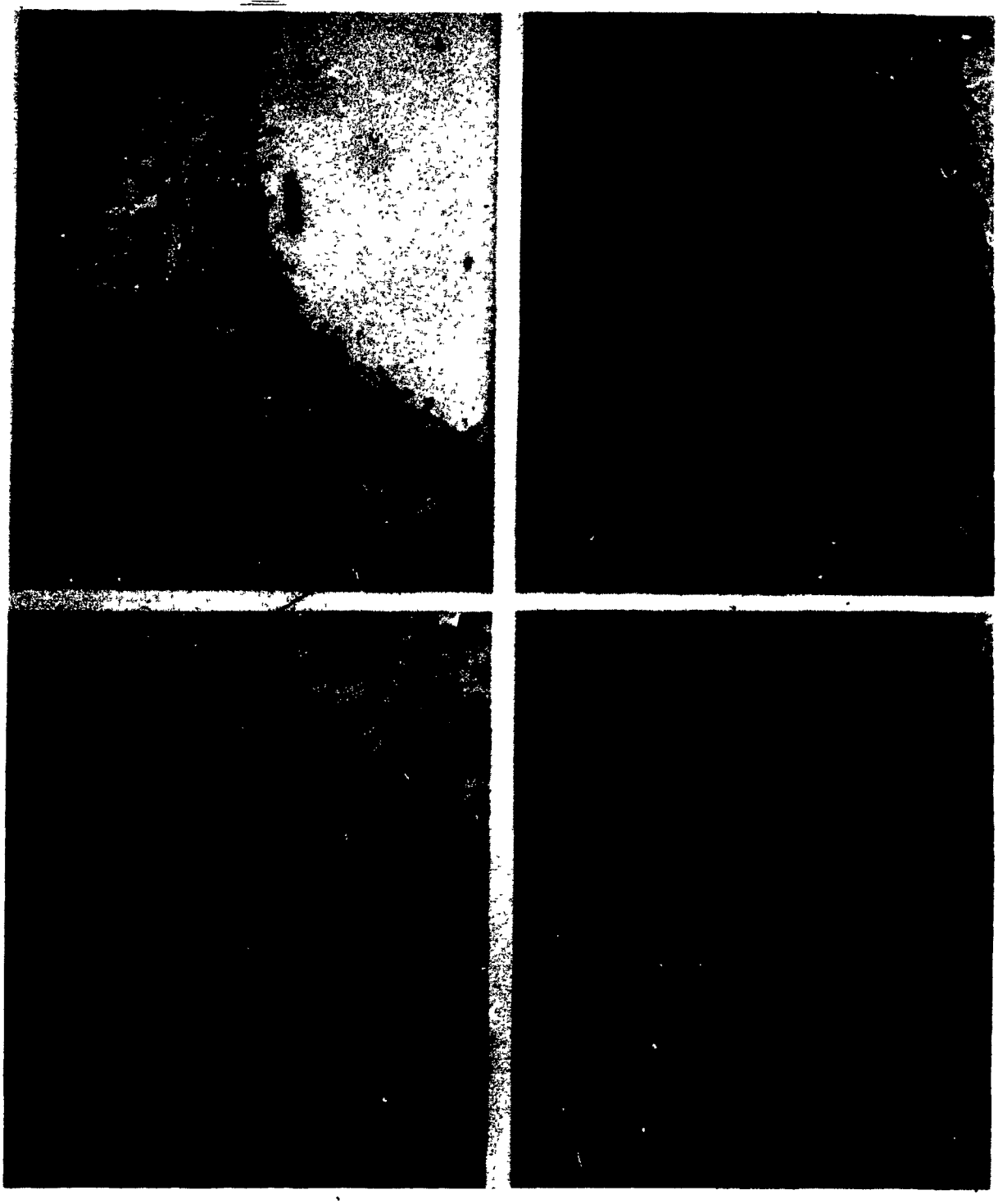


FIGURE 24

Labyrinthine region of a heterozygous placenta at 15 min. In the trichorial labyrinth (layer 1, 2 and 3), membrane bound silver grains are located in layer 3 (arrows) (x 6,000)

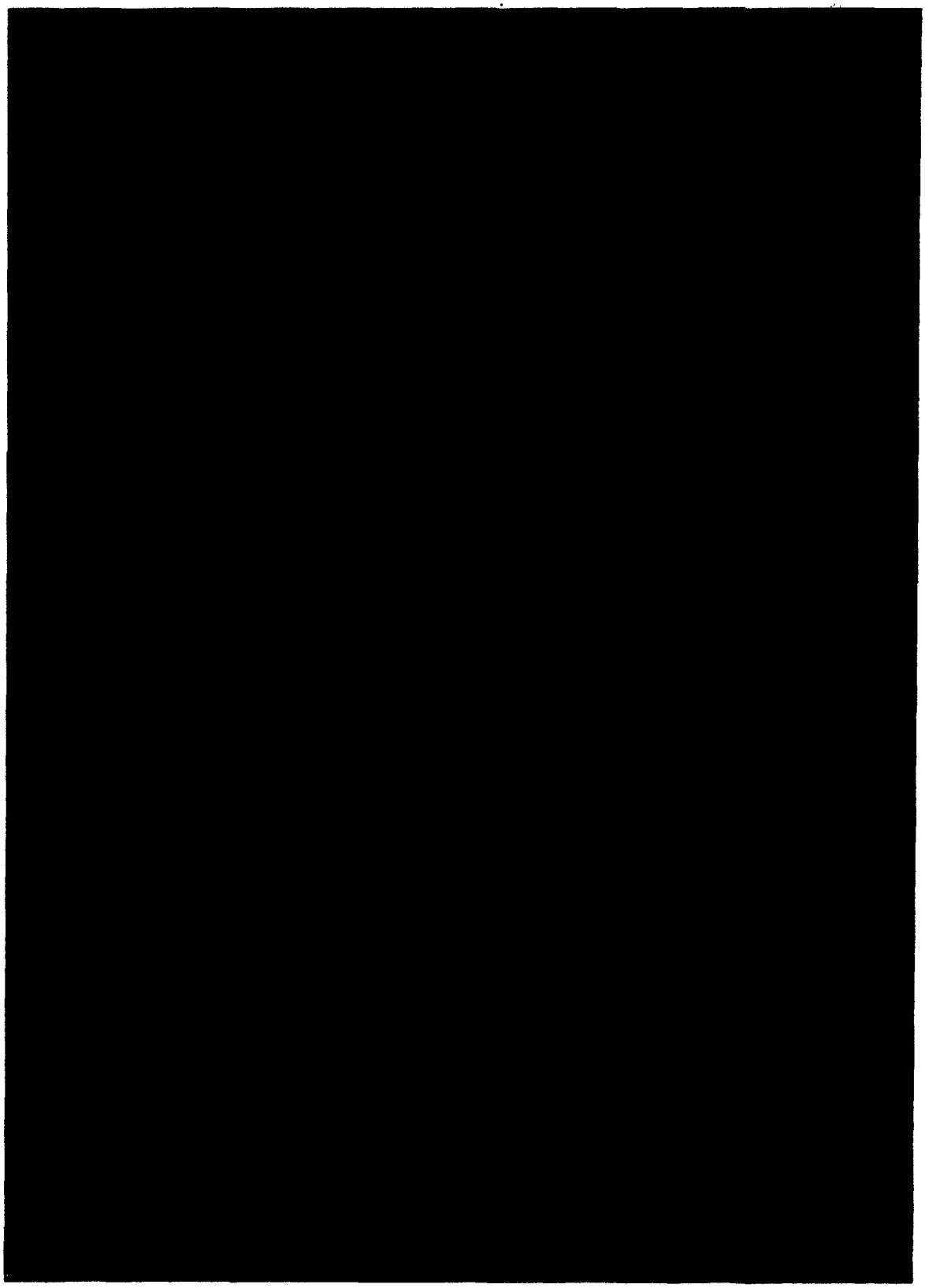


FIGURE 25

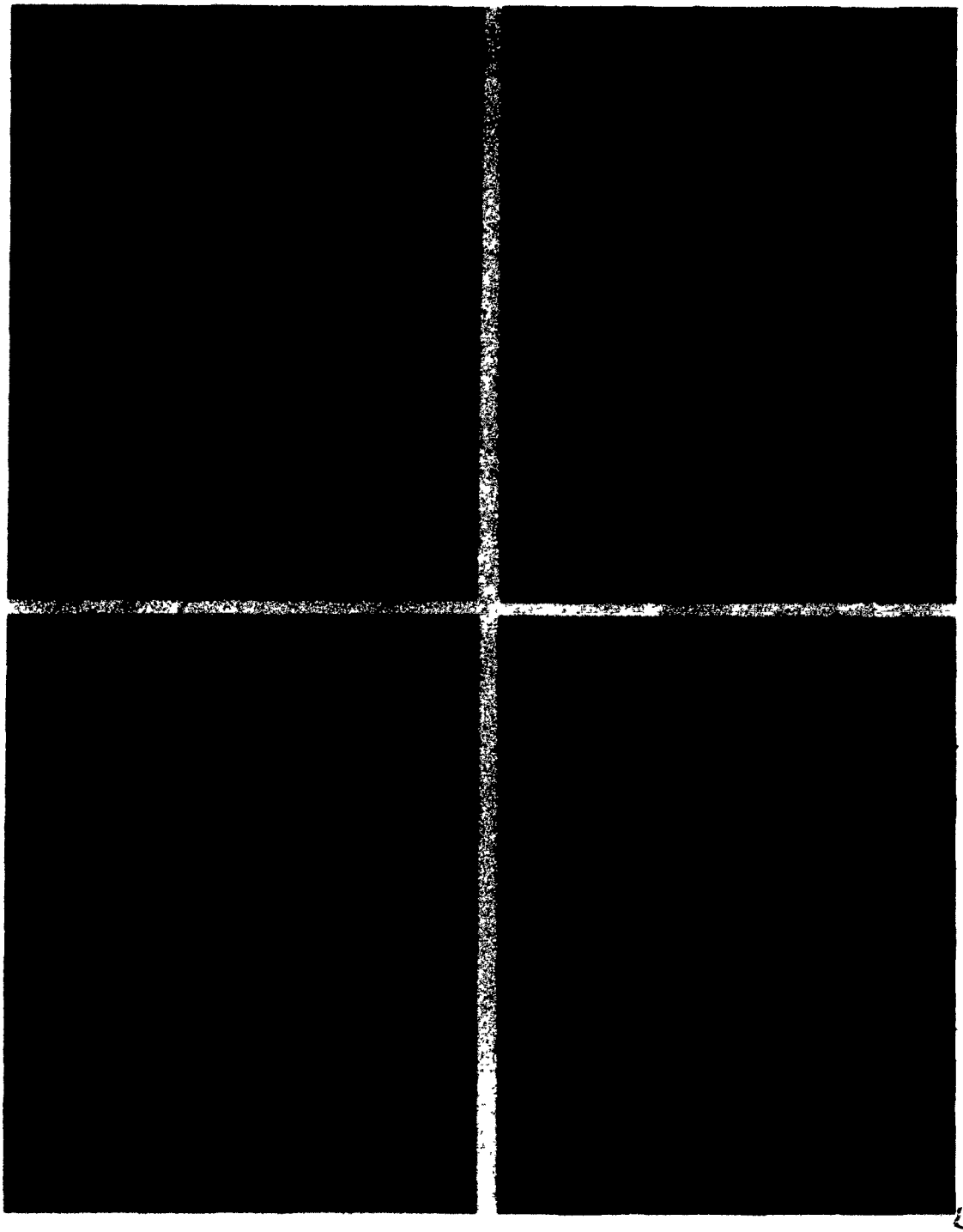
Labyrinthine region of heterozygous placenta at 1 hr

(a) A macrophage type cell (M) in the interstices of the labyrinth shows plasma membrane bound as well as intracellular labeling (x 2000)

(b) Part of a labyrinthine trophoblast cell in the deepest (3rd layer) of the labyrinth showing plasma membrane bound (arrowhead) as well as intracytoplasmic label associated with lipid vesicles (arrows) (x 14,000)

(c) Shows autophagic vacuoles (arrows) associated with labyrinthine trophoblast cells containing labeled materials (x 2,000).

(d) A higher magnification of the upper right vacuole in (c) showing the localization of the label on various membranous structures in the process of autophagy (x 14,000).



class 1 antigens of the paternal haplotype in situ.

At later time points, particularly at 1 hour, labeling was seen on the cell membrane as well as at intracellular locations on all types of labyrinthine trophoblast cells and monocyte-macrophage class of cells within the labyrinth. Labeling was also localized in autophagic vacuoles suggestive of degradation of membranes and the associated membrane bound label (Figure 25).

3.6.2 Spongiotrophoblast Region

In concordance with the light microscopic results, spongiotrophoblast cells of the heterozygous placenta demonstrated significant labeling at 30 minutes onwards. The labeling was initially localized on the sinusoid lining microvillous plasma membrane (Figures 26 and 27). Homozygous spongiotrophoblast cells did not reveal significant labeling. Thus, spongiotrophoblast cells also express, in situ, paternal H-2 antigen on the sinusoid lining plasma membrane. At 1 hour, in addition to a strong labeling of the plasma membrane, intracellular transport of the label is also observed (Figure 28).

3.6.3 Fetal Stromal Cell Clusters

Fetal stromal cells, as described earlier, are usually surrounded by a mantle of spongiotrophoblast cells extending into the labyrinthine zone. These cells in the heterozygous placenta exhibited the strongest labeling at the light microscope level.

The labeling pattern of these cells at the ultrastructural level is illustrated in Figure 29. Moderate labeling on the plasma membrane of these cells was observed as early as 2 minutes in the heterozygous

FIGURE 26

Spongiotrophoblast cells of the heterozygous placenta at 30 min. showing labeling (arrows) of the sinusoidal plasma membrane associated with microvilli (x 14,000)

S = Maternal Sinusoid

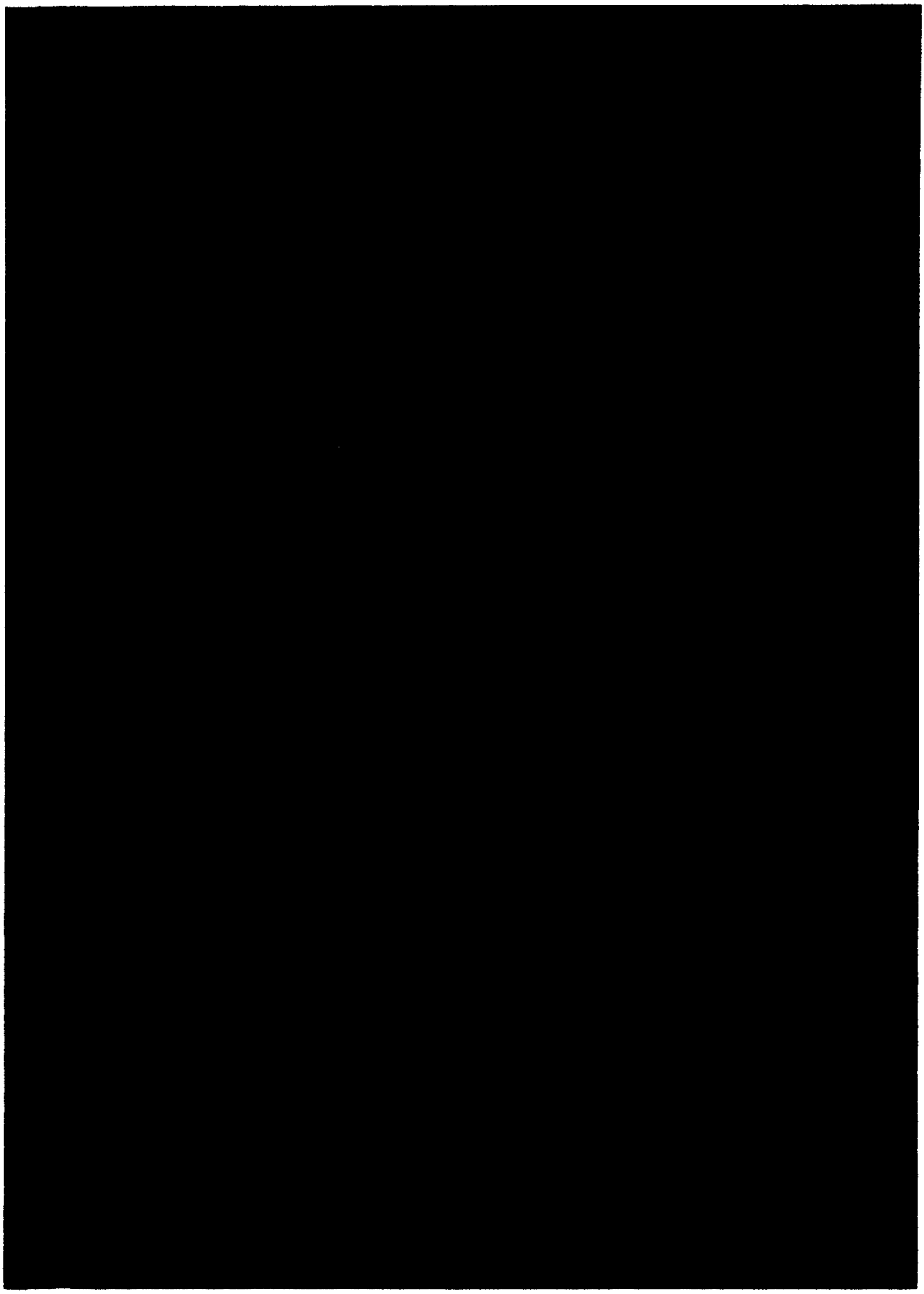


FIGURE 27

A high magnification of the junctional point of two sinusoid lining spongiotrophoblast cells of heterozygous placenta at 30 min. Note labeling of microvilli at the sinusoidal face of the plasma membrane (arrows). Cross section of a microvillus (arrowheads) is hidden under dense silver grains (x 24,000)

S = Maternal Sinusoid

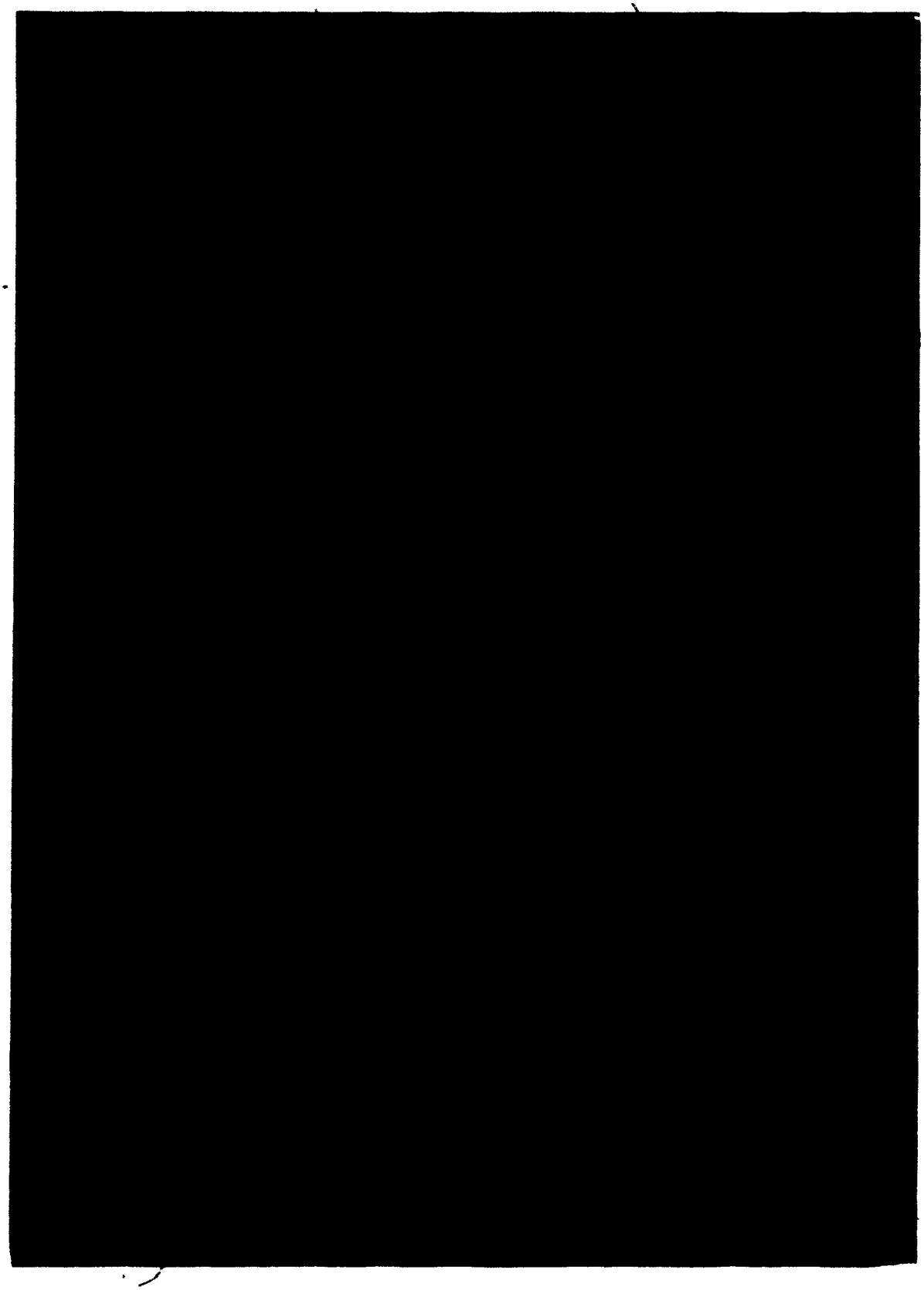
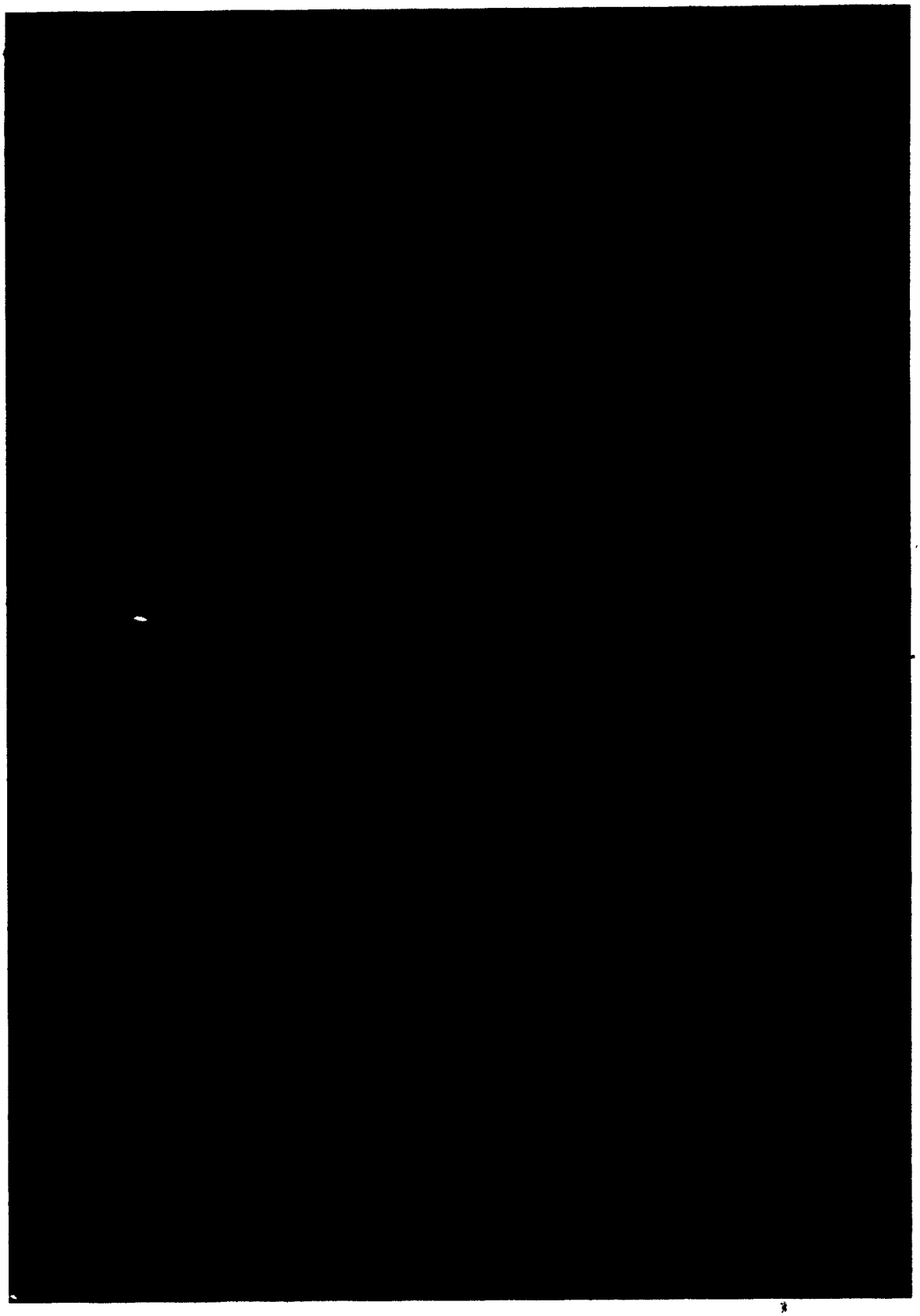


FIGURE 28

Labeling of a heterozygous spongiotrophoblast cell at 1 hour. Labeling is seen on the microvilli (arrow) as well as within the cytoplasm (arrowheads) (x 2,700)

S = Maternal Sinusoid

C = Fetal Capillary



not reject the conceptus. (2) Certain MHC epitopes on trophoblast cells recognized by B cells may not be recognized by T cell's (Chatterjee-Hasrouni and Lala 1982). This hypothesis remains to be tested by use of multiple monoclonal antibodies and alloreactive T cell clones. (3) A local immunoregulation by products of certain cells at the fetomaternal interface including trophoblast cells themselves (Pavia and Stites 1981; Chaouat and Kolb 1984), or maternally derived cells in the decidual tissue eg. certain suppressor lymphocytes of the null phenotype (Slapsys et al 1983) and typical stromal type decidual cells (Lala et al 1983, 1984; Parhar and Lala 1985 Lala et. al 1985) The latter studies have demonstrated conclusively that decidual cells strongly suppress mixed lymphocyte reaction in vitro and prevent the generation of alloreactive killer cells. This suppression is mediated by prostaglandins, primarily PGE , in an MHC nonspecific manner. It remains to be tested whether this suppression also applies to a secondary response. A similar prostaglandin mediated local suppression exerted by decidual cells on the generation of functional development of the NK lineage cells within the decidua has also been identified (Scodras et al 1985). Decidua is found to be infiltrated with a large number of null lymphocytes (Kearns and Lala, 1985a) many of which have been identified to be of the NK lineage capable of recognizing target structures on trophoblast cells (Chatterjee-Hasrouni et. al, 1984). However, these cells are rendered inactive. The progesterone mediated suppression by the trophoblast and prostaglandin mediated suppression by the decidual cells may jointly represent the most potent immunoregulatory phenomenon at the fetomaternal interface. A

background despite the presence of Fc receptors on these cells, indicating that antigen-antibody complexes from the placenta are not transferred to these cells.

Yolk sac entodermal cells never showed specific labeling at any time point even when labeling was observed on Reichert's membrane and macrophages located within the yolk sac cavity (Figure 31). Therefore, yolk sac cells do not appear to express paternal haplotype H-2 antigens on any part of their cell membrane - whether apical or basolateral.

Reichert's membrane showed some labeling in both placental types at all time points. The mechanism of this labeling is unknown at present.

FIGURE 29

Presents labeling associated with fetal stromal clusters (see section 3.1 for a light microscopic description of these cells) in the labyrinthine region of the heterozygous placenta at different time intervals.

(a) At 2 min: Note moderate labeling of the plasma membrane of these cells (x 2,000)

(b) and (c) at 1 hr. Note heavy labeling of the plasma membrane and some minor intracellular labeling (x 2,000).

(d) and (e) are higher magnifications of the sections marked 1 and 2 of (c) respectively (x 8,000)

S = Maternal Sinusoid

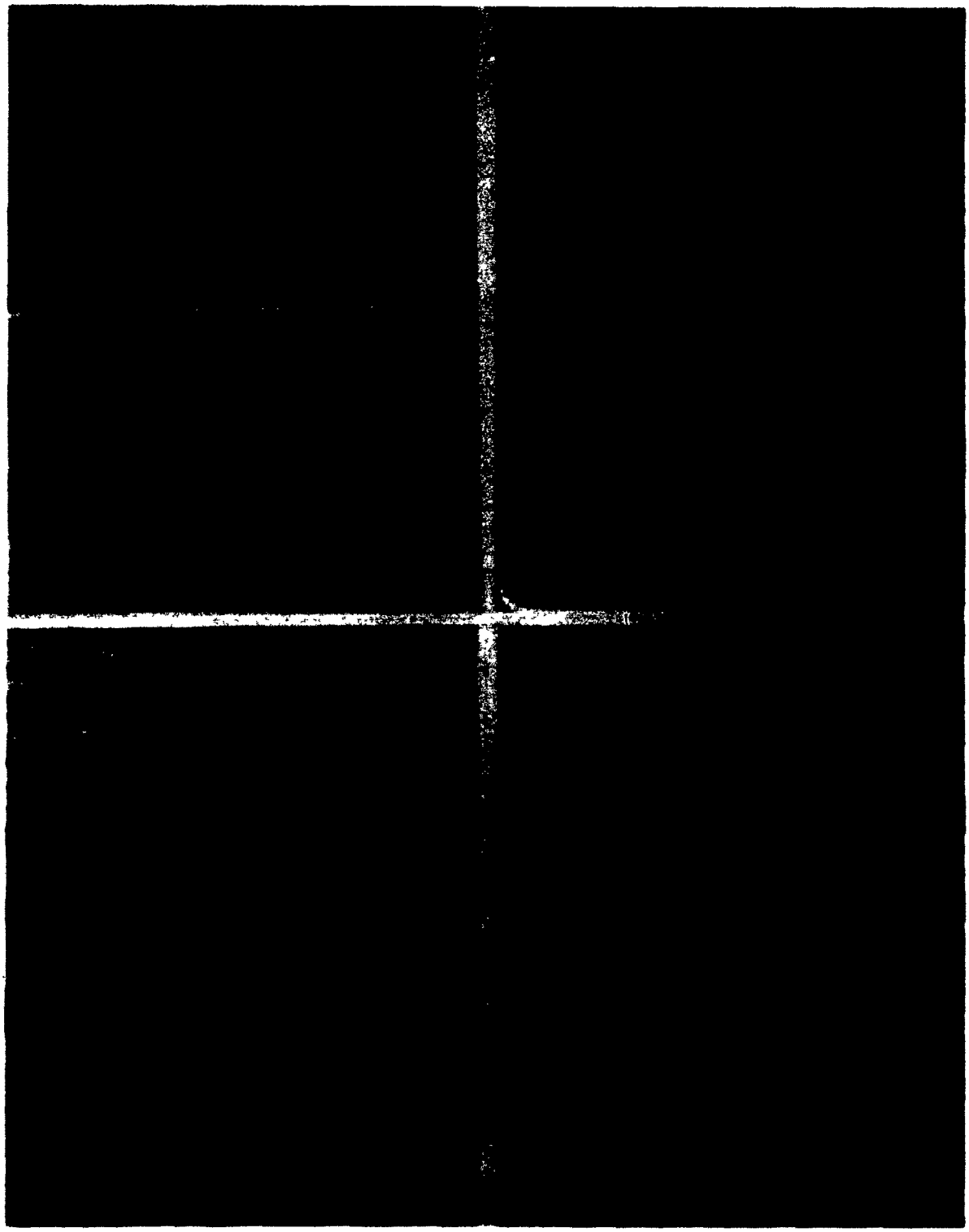


FIGURE 30

Shows labeling of fetal endothelial cells (a & b) and a macrophage (c) in the labyrinthine region of a heterozygous placenta.

(a) Labeling is shown on the fetal endothelial cell plasma membrane (arrows) at 2 min. A few silver grains appearing on the adjacent labyrinthine trophoblast cell nucleus is possibly due to the latent image formed at a distance from the radiation source of I^{125}, which has a high energy range (x 5,000)

(b) Another endothelial cell showing silver grains on the plasma membrane (arrows) at 1 hr. Some intracellular labeling is seen in the adjacent trophoblast cell (x 10,000).

(c) A macrophage located within the interstices of the labyrinth showing plasma membrane labeling (arrowheads) at 15 min. Some intracytoplasmic labeling is observed in the adjacent trophoblast (x 5,000)

C = Fetal Capillary

EC = Fetal Endothelial Cell

M = Macrophage

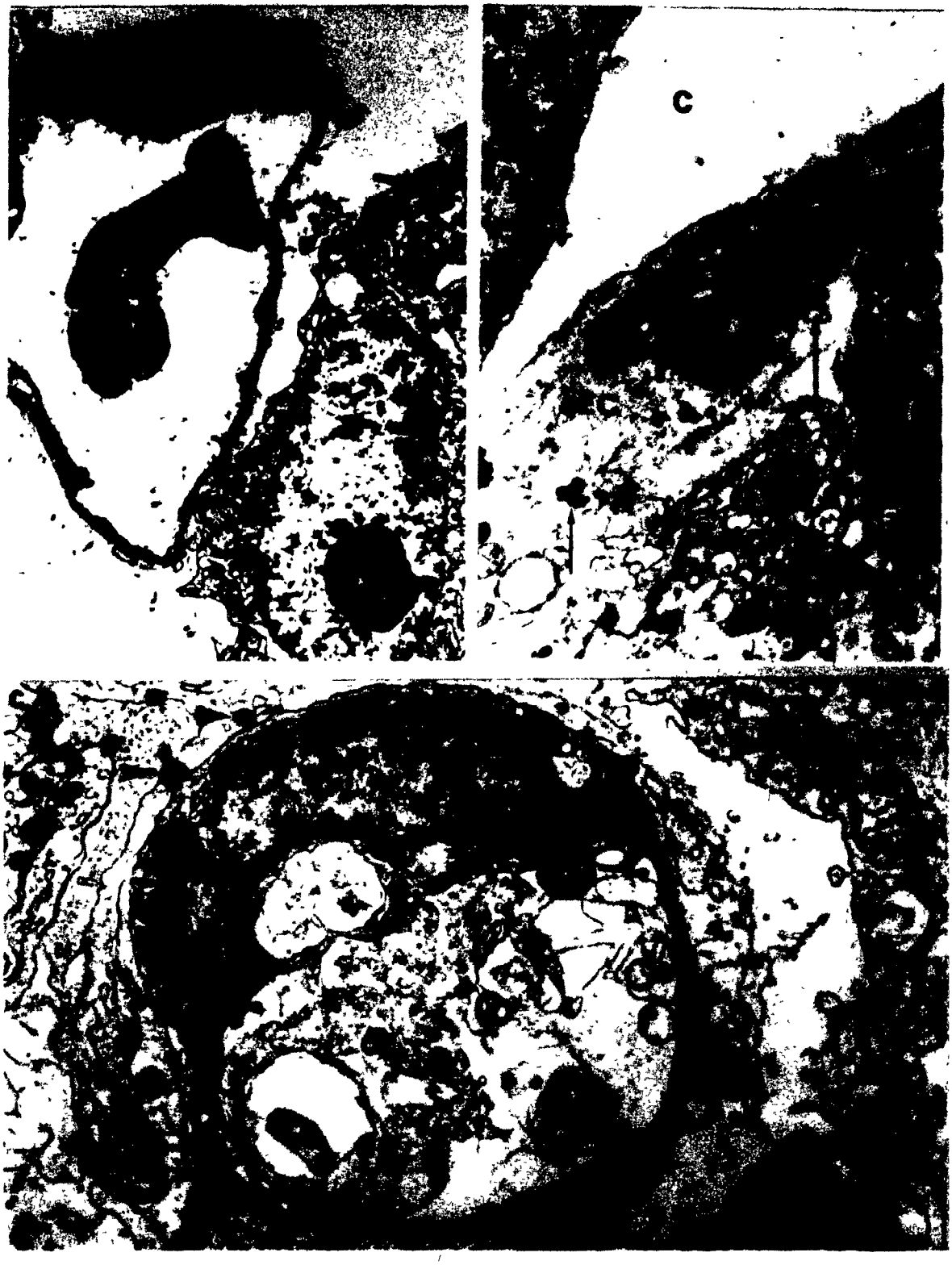

c

FIGURE 31

Labeling of the heterozygous placenta at 1 hour.

(a) Yolk sac region showing nonspecific labeling of the Reichert's membrane. Entodermal cells do not show any significant labeling (x 5000).

(b) Giant trophoblast cell showing no labeling (x 2000).

(c) Decidual cells do not show any labeling (x 5000).

RM - Reichert's Membrane
GC - Giant Trophoblast Cell
DC - Decidual Cell
En - Yolk Sac Entodermal Cell.

DISCUSSION

The present study was designed primarily to establish the precise identity of various cell types of fetal origin within the 15 day murine placenta, which express class 1 MHC antigens of the paternal haplotype *in situ*, as well as the precise location of the antigen bearing sites. These objectives were achieved respectively with light microscope and electron microscope radioautography. The latter technique, in addition, provided some knowledge about the intracellular movement (transport and/or processing) of labeled molecules with time following the initial labeling of the plasma membrane.

A quantitative analysis of the temporal pattern of labeling intensity for various cell types in the heterozygous versus homozygous placentae at the light microscope level permitted an evaluation of the relative antigen density on the antigen bearing cells which had access to the antibody at various time points, and the degree of antibody binding via Fc receptors. H-2 related vs Fc receptor related labeling in the placenta was further evaluated with the aid of *in vitro* labeling experiments. The temporal pattern of the overall retention of radioactivity in the placenta as compared to the fetus permitted a testing of whether the placenta served as a selective immunoadsorbent barrier to the passage of antipaternal type H-2 antibody molecules to the fetus.

Studies of antibody binding *in vivo* and *in vitro* employed whole intact antibody molecules (IgG2b subclass) rather than their $F(ab)_2$ fragments to mimic the physiological situation in which the pregnant mother usually produces IgG (IgG1 and IgG2) type antifetal MHC

antibodies (Bell and Billington, 1983) in the circulation. Blood clearance curves (Figure 7) when compared with the whole placenta radioactivity curves (Figure 8) demonstrated that at very early intervals (2-15 minutes), a minor amount of blood contamination in the perfused placentae may have significantly influenced the values of the whole placental radioactivity. For this reason, later intervals (30 minutes - 6 hours) were used for a comparison of the ratios of radioactivity retained per unit mass of the placenta to that of the fetus during homozygous versus heterozygous pregnancy (Figure 9). These ratios were identical in both types of pregnancies except at the 30 minute interval. Although we have not investigated the molecular nature of the fetus-bound radioactivity, these results provide no concrete evidence for a selective immunoadsorbent barrier function of the heterozygous placenta to the passage of antifetal type class 1 MHC antibody molecules to the fetus. Recently Adeniyi-Jones and Ozato (1984), using a similar combination of H-2 genotypes, gestational age (15 days) and an intravenous administration of radiolabeled monoclonal anti-H-2k^k antibody reactive against the paternal haplotype, but different time intervals (24 to 72 hours post injection) showed that undegraded labeled antibody molecules crossed the placenta to appear in the heterozygous fetus in the same proportion as the homozygous fetuses. Both their and our results are in conflict with those reported by Wegmann's group (Wegmann et al., 1979; Raghupathy et al., 1981) who noted a preferential retention of antipaternal type H-2k^k antibody in the heterozygous (BALB/c ♀ x C3H ♂) placentae at 2-24 hours post injection of the antibody. Whether the disagreement in these

results are related to the difference in the mouse strains employed or some other factor(s) remains undetermined at present. It must be pointed out, however, that only a minor fraction (6-25%; Figure 9) of the placenta bound label crosses to the heterozygous and homozygous fetuses indicating that both types of placentae act as efficient barriers to the passage of antibody molecules to the fetus in an H-2-nonspecific manner.

Results of the in vitro labeling experiments, using dispersed homozygous and heterozygous placentae, permitted several conclusions: 1) there is a significant incidence of Fc receptors on cells of both placental types, 2) this incidence is identical for both placental types, and 3) the affinity of antibody binding to the H-2 antigens is stronger than to the Fc receptors at both 4°C and 37°C. These findings allow for a better interpretation of the results on temporal changes in the labeling intensity of various placental cell types at the light microscope level. Thus, any in vivo labeling above background noted on cells within the homozygous placenta provided a relative measure of the antibody binding via Fc receptors. Since the incidence of Fc receptors was identical for both placental types, the labeling intensity of the individual cell type within the heterozygous placenta over and above that in the homozygous placenta provided a relative measure of the H-2 related binding. The latter, in turn, could result from the antibody binding to H-2 antigenic sites at all intervals or a binding of antigen-antibody complexes to Fc receptors at later intervals.

Following the administration of the radiolabeled antibody, labyrinthine trophoblast cells were the earliest to show significant H-2

specific labeling in vivo in the heterozygous placenta. Little Fc related labeling was seen on these cells in the homozygous placenta. Thus, the differential labeling noted in the former case must be due to the presence of H-2 antigenic sites. This finding confirms the results reported earlier from this laboratory by Chatterjee-Hasrouni and Lala (1982). They observed a preferential labeling of the sinusoidal face of the heterozygous labyrinthine trophoblast cells at 15 minutes following the injection of antipaternal type H-2 antibody via the placental artery. However, this study as well as our results at the light microscope level could not conclusively discard the possibility of a sequestration of the antigenic sites to the basolateral face of the plasma membrane of the labyrinthine cells constituting the first layer of the trichorial labyrinth, nearest to the maternal sinusoids. Present radioautographic studies at the electron microscope level definitely excluded this possibility. The results revealed a rapid, sequential H-2 specific labeling of the plasma membrane of all three trophoblast layers of the trichorial labyrinth, arguing in favor of an in situ expression of paternal haplotype H-2 antigens. Furthermore, such antigenic sites were first detectable on the microvilli of the sinusoid lining plasma membrane of the first layer.

The spongiotrophoblast cells, identified at the light microscope level, showed little Fc related labeling above background. Thus H-2 related labeling of these cells in the heterozygous placenta observed between 30 and 60 minutes, must be primarily due to H-2 antigenic sites (rather than antigen-antibody complexes) to which the access of the antibody was delayed because of a paucity of maternal sinusoids in the

spongiotrophoblast zone. This may explain the negative results reported by Chatterjee-Hasrouni and Lala (1982) at 15 minutes following the injection of antibody. The relative antigen density on the spongiotrophoblast was found to be lower than that on the labyrinthine trophoblast cells as revealed by a comparison of their peak labeling intensities. Electron microscope radioautography demonstrated, in addition, the expression of the antigenic sites on the sinusoid lining plasma membrane of spongiotrophoblast cells. Jenkinson and Owen (1980) also reported the presence of H-2 antigens on cultured cells of the spongiotrophoblast zone. However, the precise morphological identity of these cells could not be established by their methodology. Similarly, Singh et al (1983) reported in vivo H-2 related labeling of cells of spongiotrophoblast zone at a late interval, which they tentatively identified as spongiotrophoblast cells. However, the morphological features of the labeled cells in the published radioautograph appear, to us, similar to those of fetal stromal cell clusters, which label strongly in our hands as discussed later.

Giant trophoblast cells examined at the light or electron microscope level were negative for H-2 specific labeling at any time interval, confirming the negative results documented by Chatterjee-Hasrouni and Lala (1982).

Strong H-2 related labeling was observed on fetal stromal cell clusters. These cells also revealed significant Fc related labeling. It is highly likely that the H-2 related labeling of these cells at later time intervals was the combined result of antibody binding via H-2 antigenic sites and a binding of antigen-antibody complexes via Fc

receptors. Thus, these cells are strong candidates for the immunoadsorbent function in the placenta, although this function may not be selective for antifetal type antibodies. An ultrastructural examination of these cells clearly demonstrated that most of the binding by either mechanism mentioned above was confined to the plasma membrane.

All the conclusions made above for the fetal stromal cells also apply equally to the endothelial cells of fetal capillaries. Furthermore, an H-2 specific labeling of their plasma membranes as early as at 2 minutes indicates a very rapid transfer of the antibody to these cells from the maternal sinusoids. This labelling was usually found to be confined to the abluminal plasma membrane wherever this membrane was separated from the luminal plasma membrane by the nucleus. When the two plasma membranes were too close together, the weak resolution of the filamentous silver grains did not permit a precise localization on one or the other membrane (Figure 30).

Macrophage labeling under the light microscope was both FcR and H-2 related. H-2 related labeling of these cells was the strongest amongst all placental cells at 2 minutes (23.6 grains for the heterozygous cells as compared to 6 grains per unit area for the homozygous cells as shown in Table 1). This finding can be best explained on the basis of high density paternal type H-2 antigens on their surface, denoting that these cells were most likely fetal in origin. However some high affinity FcR binding of antigen-antibody complexes released from other cells or their internalization could not be totally excluded even at early time intervals, since

both membrane bound and internalized grains were a common occurrence in these cells at the ultrastructural level as early as at 5 minutes interval. These were the only cell type in which intracytoplasmic labeling included lysosomes, suggesting that they are involved in the degradation of antibody or antigen-antibody complexes. Other forms of intracytoplasmic labeling of various structures such as coated vesicles, and lipid vesicles were common to all cell types, particularly at later intervals, indicating a transport of labeled molecules. In addition, autophagic vacuoles containing membrane bound label was seen within trophoblast cells suggesting a degradation of membranes which have bound the antibody or antigen-antibody complexes. A further quantitative analysis of radioautographs at the ultrastructural level at frequent time intervals employing fine grain development after an administration of radiolabeled whole antibody as well as $F(ab)_2$ fragments, combined with a molecular analysis of label in the placenta and fetus remain as future considerations. Such analysis should provide a clearer picture of the intracellular transport, processing and the fate of the antibody and antigen-antibody complexes while crossing the placental barrier.

A lack of labeling of the yolk sac entodermal cells at the light and electron microscope levels confirms the absence of H-2 antigens on these cells reported by Parr et al (1980). However, Reichert's membrane showed significant labeling above background in both placental types at the light and electron microscope levels at all time points. Although a binding of antigen-antibody complexes to basement membranes in certain structures such as renal glomeruli is a common feature under

certain pathological conditions, such a phenomenon alone cannot explain the current findings on the Reichert's membrane, since significant labeling also occurred in the homozygous placenta.

The present study is the first formal demonstration of the presence of H-2 antigenic sites on the murine trophoblast plasma membrane exposed to the maternal sinusoid. Moreover, we have also located such sites on spongiotrophoblast plasma membrane facing the maternal decidua, which is known to be infiltrated with cells of the maternal immune system (Kearns and Lala, 1985a). Similarly, human cytotrophoblast cells embedded in the maternal decidua (Sunderland et al 1981; Montgomery and Lala, 1983) as well as located on the basal plate (Wells et al, 1984) express class 1 MHC antigens. Yet, there is no evidence for an allograft response of the otherwise immunocompetent mother against trophoblast cells. There is ample evidence for a cognizance of these antigens by the maternal B cells with the production of antibodies, but a T cell response leading to a generation of killer cells has not been demonstrated at the fetomaternal interface.

Several explanations for the above paradox can be offered: (1) An absence of class 2 MHC antigens on the trophoblast cells (Chatterjee-Hasrouni and Lala 1981; Montgomery and Lala, 1983) despite the presence of class 1 antigens may make these cells poorly immunogenic like thyroid parenchymal cells (Lafferty and Woolnough, 1977). However, class 2 antigens are considered to be important for a primary and not a secondary allograft response, whereas a mother presensitized to fetal antigens still does

not reject the conceptus. (2) Certain MHC epitopes on trophoblast cells recognized by B cells may not be recognized by T cells (Chatterjee-Hasrouni and Lala 1982). This hypothesis remains to be tested by use of multiple monoclonal antibodies and alloreactive T cell clones. (3) A local immunoregulation by products of certain cells at the fetomaternal interface including trophoblast cells themselves (Pavia and Stites 1981; Chaouat and Kolb 1984), or maternally derived cells in the decidual tissue eg. certain suppressor lymphocytes of the null phenotype (Slapsys et al 1983) and typical stromal type decidual cells (Lala et al 1983, 1984; Parhar and Lala 1985, Lala et. al 1985) The latter studies have demonstrated conclusively that decidual cells strongly suppress mixed lymphocyte reaction in vitro and prevent the generation of alloreactive killer cells. This suppression is mediated by prostaglandins, primarily PGE_2, in an MHC nonspecific manner. It remains to be tested whether this suppression also applies to a secondary response. A similar prostaglandin mediated local suppression exerted by decidual cells on the generation of functional development of the NK lineage cells within the decidua has also been identified (Scodras et al 1985). Decidua is found to be infiltrated with a large number of null lymphocytes (Kearns and Lala, 1985a) many of which have been identified to be of the NK lineage capable of recognizing target structures on trophoblast cells (Chatterjee-Hasrouni et. al, 1984). However, these cells are rendered inactive. The progesterone mediated suppression by the trophoblast and prostaglandin mediated suppression by the decidual cells may jointly represent the most potent immunoregulatory phenomenon at the fetomaternal interface. A

synergistic action for the two molecules has been demonstrated in vitro (Fujisaki et al, 1985), against lymphocyte activation. Thus, another form of potential assault on the conceptus is averted by the decidual cells which have now been recognized as a class of marrow derived immunoregulatory cells having multiple functional potential (Lala et al 1985).

Original Contributions

(1) A demonstration in the 15 day murine placenta of the presence of paternal haplotype class I MHC antigens in situ on the plasma membrane of all three trophoblast layers of the trichorial labyrinth and spongiotrophoblasts, some of the antigenic sites being located on the sinusoid lining microvilli. Antigenic sites were also demonstrated on fetal stromal cells, capillary endothelium and macrophages, which also bore Fc receptors.

(2) Identity of a unique cell class of fetal origin, occuring in clusters within a spongiotrophoblast mantle located mostly in the labyrinthine zone have been established for the first time in the murine placenta. These cells bear FcR, and may play an important role in trapping Ab and Ag-Ab complexes.

(3) (C57BL/6 ♀ x CBA ♂) placentae do not appear to exert a selective barrier function against the passage of antifetal type H-2 antibodies to the fetus.

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