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Immunological Considerations at the Maternal-Fetal Interface: The Placental Barrier to Cellular Traffic and Nature of the Antigens Involved

by

Rosemarie D. Hunziker

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Submitted to the Faculty of Graduate Studies and Research
in partial fullfilment of the requirements for the degree of

Doctor of Philosophy

in

Medical Sciences (Immunology)

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NAME OF AUTHOR Rosemarie D. Hunziker

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(Signed) Rosemarie D. Hunziker

Permanent Address:

11519 Running Cedar Road
Reston, Virginia 22091
U.S.A.

Dated September 1987

Preface

Parts of this work have previously appeared in *The Journal of Immunology* and *Critical Reviews in Immunology*.

The University of Alberta
Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled *Immunological Considerations of the Maternal-Fetal Interface: The Placental Barrier to Cellular Traffic and Nature of the Antigens Involved* submitted by Rosemarie D. Hunziker in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Medical Sciences (Immunology).

T. Mosmann
Supervisor

Bhagwan Singh

Ann Tucker

A.S. Russell (on sabbatical)
for

Judith R. Head
External Examiner

Date 10/13/87

Dedication

This thesis is dedicated to:
Hans and Stella,
who brought me to the precipice,
Susan,
who pushed me over, and
Marcus,
who gave me a parachute.

Abstract

The intimate juxtaposition of maternal and embryonic tissues throughout mammalian gestation poses the question of fetal survival within an immunologically intact semiallogeneic mother. The placenta excludes potentially alloreactive cells from access to the fetus. Using electrophoretic resolution of the two allelic variants of the ubiquitous enzyme, glucose phosphate isomerase, we examined fetal tissues for the presence of maternal cells. Only two of 172 animals assayed contained maternal isozyme. Since the detection limit was of 1% of the minority (maternal) isozyme, we continued our investigations using a more sensitive method, the examination of fetal tissues for the presence of fluoresceinated cells previously injected into the maternal circulation. While RBC can cross the placenta in low numbers, only a very few maternal leukocytes were found in the fetal liver.

It was not clear if the placental barrier was a purely physical one, or if trophoblast antigens selectively absorbed potential anti-fetal effectors. Therefore, we investigated the ontogeny and distribution of class I antigens in gestational tissues.

Pre- and peri-implantation embryos reacted with monoclonal antibodies directed against beta$_2$ microglobulin, the invariant light chain of class I antigens. However, embryos from three different haplotypes did not bind to the relevant monoclonal antibodies specific for the class I heavy chain. This indicated the presence of non-traditional class I antigens on early embryos.

Genes located in the major histocompatibility complex (MHC), near the polymorphic and nearly ubiquitously expressed traditional class I antigens, encode class I genes of limited polymorphism and distribution. We investigated the possibility that these Qa and Tla region genes produce embryonic class I. Two different isotype matched monoclonal antibodies, reacting with either traditional class I determinants only or traditional plus Qa antigens were radiolabeled and administered to pregnant mice, and the levels of antibody bound to gestational tissue determined. There was no difference in the binding patterns of the two antibodies, no evidence for expression of non-traditional class I antigens.

Steady state levels of specific mRNA can be predictive of protein expression. Using a set of well-defined oligonucleotide probes, we showed

that the traditional class I genes were expressed in mid- to late gestation placenta, yolk sac and embryos. The genes neighboring K and D are also expressed. None of the probes derived from the Qa region bound to the RNA, but a few Tla region probes showed weak hybridization.

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Introduction

The intimate juxtaposition of maternal and embryonic tissues throughout mammalian gestation poses the question of fetal survival within an immunologically intact semiallogeneic mother (Little, 1924). In 1953, Medawar proposed potential solutions to this problem. Billingham (1964) and Simmons (1969) elaborated these solutions into testable hypotheses and summarized earlier work. The possible mechanisms included: a) the uterus is an immunologically privileged site, b) the conceptus is antigenically immature, c) the placenta provides a barrier to cellular and humoral maternal-fetal interactions, d) the mother is immunosuppressed, and e) the immune response of the mother is qualitatively different during pregnancy, and promotes rather than hinders fetal survival. Over the span of three decades we have managed to eliminate only the first option (Beer and Billingham, 1974, 1976); there is evidence in partial support of all others. This review will summarize these studies concerning the "riddle of the fetal allograft" (Chaouat, 1985) with special emphasis on the display of allo- and stage specific transplantation antigens at or near the maternal-fetal interface.

A. Ontogeny and Morphology of the Placenta and Fetal Membranes

Immunological processes require recognition of antigen by cell surface receptors. In the maternal-fetal relationship this implies a confrontation of maternal immunocompetent cells with semiallogeneic fetal tissue at a boundary. This interface is the trophoblast--extra-embryonic tissue that is first isolated from the embryo proper at the blastocyst stage early in development. Trophoblast, in its various differentiated forms, is the fetal tissue which abuts maternal surfaces at all points of contact between the two. It interposes a contiguous layer between the two circulatory systems in the placenta, a barrier between maternal endometrial tissue and the outer areas of the placenta, and, at least throughout most of gestation, a layer surrounding and separating the extraembryonic membranes from the uterine wall. Indeed, Faulk and McIntyre (1983) note that the trophoblast, and not the fetus, is the allograft of pregnancy. Sensitization of the mother can occur at the various contact sites,

specifically decidual tissue, uterine endometrium, endothelium of the spiral arteries, and maternal blood-borne lymphocytes within them, or throughout the maternal circulation, since pieces of trophoblast break away from the placenta and enter the maternal blood vessels (Douglas *et al.*, 1959 and Atwood and Park, 1961).

The placenta is a chimeric organ. Cells of the fetal villi, composed of the endothelium of blood vessels surrounded by a stromal or connective layer and overlaid with trophoblast, interdigitate with layers of maternal epithelium, stroma, and vessel endothelium. The trophoblast is invasive, and erodes successive layers of maternal tissue in forging the morphology of the placenta. The most widely used classification system for eutherian placentas is based upon the extent of this erosion (Grosser, 1927 cited in Ramsey, 1982). Since the gestational immunology of mice, rats, and humans is the best understood and is the focus of most investigators to date, the types of placentas found in these genera are of most interest here. All three groups display hemochorial placentation, in which the trophectoderm penetrates through the maternal endothelial lining of the uterine spiral arteries, thus directly exposing fetal tissues to maternal blood. The fine structure of the trophoblast sheath in the mature placenta, however, is very different in rodents which have a trilaminate arrangement (an outer cellular layer and two underlying syncytial sheets) and humans with only a continuous syncitial trophoblast and scattered cytotrophoblast cells beneath (Enders, 1965 and Hernandez-Verdun, 1974).

An in-depth description of the ontogeny of the placenta and fetal membranes will not be attempted here; the reader is refered to several excellent reviews (Amoroso, 1961; Snell and Stevens, 1966; Rugh, 1968; Boyd and Hamilton, 1970; Theiler, 1972; Wynn, 1975, 1981; Gardner, 1975; Muntener and Hsu, 1977; Begley, Firth, and Holt, 1980 and Ramsey, 1982). The broad outlines are presented as an orientation to the discussion which follows. Upon fertilization the ovum (surrounded by its protective glycoprotein coat, the zona pellucida) undergoes four-five cleavage divisions and produces a grape cluster-like mass of small, round cells. The cells on the outside then flatten and spread, forming a unicellular, hollow, fluid-filled sphere via the establishment of intercellular tight junctions. This trophectoderm encloses the inner cells mass (ICM), the less differentiated-looking cluster of cells which amass at one pole and continue rapid division. The embryo at this stage is called a blastocyst, forming by 3-3.5 days post

conception, and has traveled down the Fallopian duct to the upper reaches of the uterus. Growth produces an increase in cell size as well as number and thus the blastocyst hatches out of the zona pellucida.

Processes of the trophectoderm cells squeeze through the epithelium and basement membrane of the uterine wall, probably aided by enzymes that degrade the extracellular matrix. As the breach in the endometrial epithelium enlarges, the fully expanded blastocyst sinks further into the endometrial stroma and thus implants within the uterine endometrial lining within the next day or so. The invasive trophectoderm continues to erode maternal stroma until the small capillaries are penetrated and the trophectoderm cells are provided with an external source of nutrients. Some signal which is related to the hormones secreted by the trophoblast, and its presence as an irritant causes the endometrial cells to enlarge and proliferate, thus giving rise to the decidua. This tissue cocoons the conceptus during early gestation and its remnants persist attached to the placenta and outer fetal membranes throughout pregnancy (Bell, 1983). Development diverges here in different species; further differentiation within the trophectocerm and inner cell mass produces the mature, multi-layered placenta, the extraembryonic membranes, and the fetus.

The decidual reaction is of greatest magnitude in those species with hemochorial placentation in which the trophectodermal invasion into the uterus is maximal. There are apparently two major waves of proliferation in rodents: just after the trophectoderm embeds within the antimesometrial uterine crypts, the neighboring stromal fibroblasts divide and differentiate into large, binucleate granular decidual cells. The development of these primary decidual cells peaks by day 8 and then regresses so that by day 12 only the thinning capsular decidua remains. Meanwhile, as the ectoplacental cone invades, decidualization has begun in the mesometrium. These cells are smaller, irregularly-shaped, uninucleate, and less granular than the antimesometrial decidual cells. Some of the cells in this area are infiltrating metrial gland cells which arise from the underlying myometrium. The development of the metrial gland cells involves enlargement and acquisition of cytoplasmic granules, and thus they are distinguished from the mesometrial decidual cells. This area also recedes during development and becomes the basal decidua. The lateral decidual cells arise between these two zones and are morphologically intermediate between basal and capsular decidual cells.

Decidualization follows a slightly different path in the human where only one (or rarely up to a few) ovum implants (Wynn, 1975). Early cellular changes are not well known due to the lack of experimental material, and so the endometrial decidual development is mostly morphologically described. The cells underlying the implantation site comprise the basal decidua which goes on to form the maternal portion of the placenta. The deep implantation of the blastocyst creates an endometrial cover, and proliferation here produces the capsular decidua which encases the embryo and its fetal membranes during early pregnancy. The endometrial stromal cells in the remainder of the pregnant uterus similarly respond and become the parietal decidua. As development proceeds, the rapidly expanding amnion abuts the smooth chorion and its capsular decidual shell. Continued growth forces these membranes into contact with the parietal decidua thus obliterating the uterine cavity. By mid-gestation, there is a further compression and fusion of these layers into the amniochorionic membrane which then directly contacts the utrerine wall. There is no metrial gland evident during human gestation, but numerous endometrial granulocytes (metrial gland cell equivalents) are scattered throughout the decidua, especially during the first trimester.

In addition to the diversity of the various decidual cells themselves, there are many other cell types identified within the decidua. Of particular interest here are lymphocytes and other cells of bone marrow origin which could have immunological functions. Kearns and Lala (1982) used radiation-induced, bone marrow-reconstituted chimeras and found that a major portion of the cells in murine deciduoma derived from the bone marrow. Kreck and Clark (1985) have shown that fluorescein-labeled bone marrow (but not spleen) cells will collect in the decidua when injected into allopregnant mice. Recent studies using reliable and ubiquitous enzyme markers, however, have shown that the bone marrow contribution is only a small fraction of the total early-gestational decidual mass (Gambel *et al.*, 1985 and Fowlis and Ansell, 1985). The cells identified by Kearns and Lala could be metrial gland cells which have been shown by morphological and immunological techniques to have lymphocyte markers (Bulmer and Peel, 1977; Stewart and Peel, 1978; Peel, Stewart and Bulmer, 1982 and Searle, Bell and Billington, 1983). Human endometrial granulocytes possess some T-cell markers (Bulmer and Sunderland, 1982, 1984; Johnson and Bulmer, 1984 and Bulmer and Johnson, 1983) and Ia positive and macrophage-like cells have been isolated

from both human (Sutton *et al.*, 1983; Bulmer and Sunderland, 1984 and Bulmer and Johnson, 1984, 1985) and mouse (Searle, Bell and Billington, 1983) decidua.

The rodent embryo develops by way of unequal mitosis, growth, extracellular fluid accumulation and isolation of certain cell populations into the egg cylinder stage (6.5-7.5 days in the mouse and 7.5-8.5 days in the rat). The ICM has been changing rapidly and can be separated into embryonic ectoderm and mesoderm (embryonic endoderm delaminates later) which will produce the embryo proper, and the forerunners of the extraembryonic membranes, the amnion, yolk sac and allantois. By this time, the trophectoderm has proliferated and differentiated into at least two morphologically different populations: peripheral giant cells with enlarged, endoreduplicated nuclei and a central diploid core of smaller mitotically active cells (Figure 1). The ectoplacental cone trophectoderm cells at the edges distal to the ICM are loosely arranged and irregularly shaped; maternal blood fills the interstices between these cells. The inner ectoplacental cone is the area of most active development and, along with some cells of the trophectodermally-derived extraembryonic ectoderm, will produce most of the trophoblast derivatives of the placenta (Papaioannoi, 1982 and Rossant and Croy, 1987). The mouse placenta is discoid and labyrinthine in structure. A cross section through the mid-term organ (Figure 2) shows the layers arranged thus: trophoblast giant cells blanket the outer edges of the spongiotrophoblast or transitional zone, a poorly vascularized area of cells and extracellular matrix. Beneath this lies the densely packed cell strands and clefts of the labyrinth. There are an outer cytotrophoblastic and two inner syncytial layers comprising the labyrinthine tubules which surround a network of fetal vessels that meet and eventually fuse into the arteries and veins of the umbilicus, the direct connections to the fetal circulation. The yolk sac surrounds the embryo and its edges insert into the underside of the labyrinthine zone. In this area the absorptive epithelial surface is increased by the development of numerous villi that face the uterine wall. Although no true choriovitteline placenta forms, the yolk sac does nourish the early embryo prior to the formation of the chorioallantoic placenta. The yolk sac continues to act as an additional route of transport to the embryo, especially for such proteins as immunoglobulins (Laliberte *et al*, 1981 and Jollie, 1985). Thus it is

the giant cells, spongiotrophoblast and yolk sac which abut materna decidual tissue, and the labyrinthine trophoblast which is bathed in maternal blood.

The ontogeny of the human placenta, fetal membranes and embryo differs from that of the rodents in several respects. After implantation the extraembryonic mesoderm forms quickly and its growth compresses the yolk (blastocoelic) cavity. Small lakes of fluid scattered throughout this mesoderm coalesce to form the exocoelom which separates the yolk sac from contact with the uterus. Rapid fluid accumulation likewise isolates the primary embryonic ectoderm from the trophectoderm, and thus the amnion (the single cell layer left apposed to the inner surface of the trophoblast) is established (Figure 3). At implantation, the trophoblast is composed of a single layer of cytotrophoblast adjacent to the yolk sac mesoderm and a surrounding amorphous area of syncytial trophoblast. Differential growth within the cytotrophoblast produces columns of cells which push through the syncytium and are eventually invaded by allantoic stroma and fetal endothelium to become the chorionic villi (Figure 4). The tips of these columns flatten out toward each other forming a cytotrophoblast shell around the conceptus. Some of these cells, the interstitial cytotrophoblasts, migrate into the decidua and a subpopulation becomes the placental bed giant cells. The final form of the human placenta is more diffuse and villous than that of the mouse; the yolk sac is not so prominent (due to its early isolation from the trophoblast) and the extra-placental maternal-fetal contacts are made by the amnio-chorion. The mature villi ultimately contain a syncytial coat and underlying cellular layer (which becomes discontinuous in late pregnancy) surrounding fetal mesenchyme-stroma and endothelium. In the human, then, the cytotrophoblast shell (and possibly some patches of the underlying syncytium) lies within the decidual mass whereas the syncytial trophoblast surrounding the villi directly encounter maternal blood. Thus, the ontogeny, morphology and physiology of the placenta is quite complex, and any investigation of immunoregulation within this organ will be equally complicated.

B. Major Histocompatibility Complex.

1. Antigens

Histocompatibility (H) antigens are, by definition, cell/tissue components that lead to rejection of tissue from a donor to a genetically different host via

immune recognition and elimination (Snell, Dausset and Nathenson, 1976). These antigens are catagorized into minor histocompatibility antigens which are largely uncharacterized cell surface molecules that stimulate slow (often indefinite) rejection, and major histocompatibility antigens which are cell surface glycoproteins responsible for rapid rejection of allogeneic grafts (Klein, 1975, 1982; Goetze, 1977; Klein, Figueora and Nagy, 1983 and Steinmetz and Hood, 1983). The major H antigens are all encoded within a single genetic region: the H-2 complex on chromosome 17 in the mouse, and the HLA complex on chromosome 6 in the human. The genetic organization in other species is less well known but probably similar (Goetze, 1977 and Klein and Figueora, 1986). The vast number of functions, both normal and abnormal, that have been mapped to the major histocompatibility complex (MHC) have produced a very complicated and confusing nomenclature which has recently been simplified by Klein and his colleagues (Klein *et al*, 1981). In brief, the many proteins encoded by the MHC are genetically, structurally and functionally related, and can be grouped accordingly (Hood, Stienmetz, and Mallisen, 1983; Steinmetz and Hood, 1983; and Flavell, *et al.*, 1986).

Class I antigens are 40-45 kd glycoproteins that are co-expressed at the cell surface with the beta$_2$ microglobulin, a 12 kd protein not encoded within the MHC (Figure 5). Each mature, membrane bound class I molecule consists of three external domains of about 90 amino acid residues (N, C1 and C2 respectively), a trans-membrane region composed of about 40 hydrophobic residues, and a cytoplasmic tail of variable length (Steinmetz, *et al.*, 1981; Hood, Steinmetz and Malissen, 1983 and Kimball and Coligan, 1983). Beta$_2$ microglobulin is noncovalently associated with the C2 domain which is the most highly conserved portion of the molecule.

These antigens are nearly ubiquitous although the overall level of their expression may vary on different cell types. For example, red blood cells, hepatocytes, neurons, muscle, and some exocrine epithelial cells are very low in cell surface class I (Parr, 1980; Parr, *et al.*, 1982; Daar, *et al.*, 1984 and Ponder, *et al.*, 1983) while hematopoietic cells express high levels of these antigens. The differences in distribution of class I molecules over the surface of cells with polarized plasma membranes can be dramatic. Parr and Kirby (1979) examined class I antigen expression in epithelial tissues that were prefixed to preserve native distribution prior to immunoperoxidase staining of the cells. H-2 determinants were found in the laterobasal membranes and not

the apical surfaces. Nevertheless, the total amount of cell surface class I characteristic of a given tissue appears to be tightly controlled. Different mice of the same strain, congenic mice of different haplotypes, and male or female syngeneic mice all express similar total amounts of H-2 molecules on splenocytes (Dower and Segal, 1985).

Class I antigens are probably the most polymorphic proteins known - there are more than 50 murine alleles (Klein and Figueroa, 1986). This fact has lead to great speculation about their function (Klein, 1975; Ohno, 1977; Hildeman, *et al.*, 1981; Scofield, *et al.*, 1982; Klein, Figueora and Nagy, 1983; and Due, *et al.*, 1986). Minimally, they are important in self-nonself discrimination and surveillance of virally infected cells by the immune system (Zinkernagel and Doherty, 1979 and Klein, 1983).

The regulation of class I antigen expression is complex. Indeed, Weis and colleagues (Weis and Seidman, 1985, and Weis and Murre, 1985) have shown that although L cells transfected with H-2 genes under the control of strong promoters express very high levels of the appropriate mRNA, total cell surface H-2 remains constant. Beta$_2$ microglobulin was not limiting in this system. Interferon can increase both class I and class II cell surface expression (Rosa and Fellows, 1984). In addition, Schrier and colleagues (1983) have shown that cultured rat cells transformed by adenovirus 12 lose class I heavy chain expression, and that this loss is correlated to malignant potential. Conversely, Moloney murine leukemia virus infection increased both class I expression and susceptibility to lysis by allospecific CTL (Flyer, Burakoff and Faller, 1985).

Class II antigens are composed of two carbohydrate bearing polypeptide chains, both of whose genes are within the MHC (Figure 5). The larger alpha subunit is 34 kd while the more polymorphic beta chain has a molecular weight of 29,000 (Hood, Steinmetz, and Mallisen, 1983 and Kaufman, *et al.*, 1984). The class II antigens are apparently restricted to B-cells, macrophages, and dendritic cells in their expression (although interferon (IFN) cannot only increase class II expression in these tissues, but also cause the appearance of Ia specificities in inappropriate tissues) and are generally somewhat less polymorphic than class I antigens. However, the combinatorial diversity available by their association within the cell membrane allows for great variability. Class II antigens are also important self markers whose major function is as presentation molecules in the triggering of immune responses;

i.e. most antigens must be presented to immunocompetent cells in association with class II molecules.

Class III antigens are proteins comprising part of the complement cascade and occasionally considered the orphan child of the MHC since they are uninvolved in immune recognition (Klein, *et al.*, 1981 and Klein, Figueroa, and Nagy, 1983). It is worth noting that there are other enzymes whose functions are unrelated to the MHC that are encoded within or near the complex.

A large number of class I-like genes are also present within the murine MHC (Figure 6), in the Qa/Tla region telomeric to the rest of the complex (Mellor, 1986; Flavell, *et al.*, 1986; and Lew, *et al.*, 1986a). Indeed, only five of the thirty-three class I genes identified in the H-2^d and three of twenty-six H-2^b class I genes (via DNA hybridization to a gene probe from a conserved region of all class I genes) map outside this area (Winoto, *et al.*, 1983; Weiss, *et al.*, 1984 and Fisher, Hunt, and Hood, 1985). Only a few of the Qa/Tla gene products have been identified and biochemically characterized, and these closely resemble the other class I antigens in structure and function (Shen, *et al.*, 1982; Michaelson, *et al.*, 1982, 1983; Boyse, 1984; Flaherty, *et al.*, 1985; Rothenberg and Triglia, 1985; McIntyre, *et al.*, 1982, Yokoyama, *et al.*, 1981; 1982 and Landolfini, *et al.*, 1985). The antigens differ from the "classical" class I antigens in that the Qa/Tla antigens are individually far less polymorphic and their distribution is restricted to certain stages of lymphocyte differentiation. The Qa subregion antigens are found only on some nucleated blood cells and their precursors (Flaherty, 1981 and Lynes, *et al.*, 1982), or as secreted products synthesized by the liver (Kress, *et al.*, 1983; Mellor, *et al.*, 1984; and Devlin, *et al.*, 1985). One popular opinion is that the genes have no essential function; that their presence on the cell surface is the result of a "loose" control on the essential expression of class I genes, and that the existance of so much class I DNA is required for the maintenance of a high level of polymorphism via gene conversion (Klein, *et al.*, 1983). Others (Flaherty, *et al.*, 1985) maintain that the Qa genes may encode essential proteins, but that the degeneracy of the genome in this region allows any of a number of structurally very similar proteins to function interchangably.

2. MHC Antigen Expression in the Embryo

A) Class I. The ontogeny of expression of MHC antigens on embryonic tissues is controversial. Specific cell surface expression of murine H-2 antigens in the preimplantation embryo has been studied using alloantisera and a wide variety of techniques including cytotoxicity (Heyner, Brinster, and Palm, 1969; Jenkinson and Billington, 1977 Krco and Goldberg, 1977), indirect immunoflourescence (Palm, Heyner and Brinster, 1971; Muggleton-Harris and Johnson, 1976 and Heyner and Hunziker, 1979), hemagglutination (Gardner, Johnson, and Edwards, 1973), ectopic transfer (Searle *et al.*, 1974), indirect immunoperoxidase (Searle *et al.*, 1976 and Billington *et al.*, 1977), indirect autoradiography (Hakansson *et al.*, 1975) and immunoprecipitation of radiolabeled proteins (Webb, Gall, and Edelman, 1977). Although there is some discrepancy as to the temporal and spacial appearance of these antigens, the general consensus is that persistent H-2 is confined to the ICM and its derivatives (Johnson, M. H., 1975; Johnson and Calarco, 1980; Bell and Billington, 1983b; Heyner, 1980 and Johnson, P. M., 1984). Attempts to repeat these experiments using monoclonal reagents have not been successful (Heyner and Hunziker, 1981) although Lala *et al.* (1984) have shown transient expression of one H-2K^k epitope on the morula using a sensitive autoradiography technique; and Warner's group has shown elevated levels of binding of pan-reactive monoclonal anti-H-2 antibodies to pre-implantation stages (Warner and Spannaus, 1984 and Goldbard, *et al.*, 1985). In addition, the *de-novo* synthesis and surface expression of beta$_2$ microglobulin (the so called "light chain" of the MHC class I molecule) can be detected in cleavage stage murine embryos (Sawicki, Magnuson, and Epstein, 1981) and blastocysts (Hakansson and Peterson, 1976). Using S1 nuclease protection assays on RNA isolated from blastocysts, Morello and colleagues (1985) could not demonstrate significant amounts of class I heavy chain message even in the presence of reduced but readily detectable mRNA for beta$_2$ microglobulin. Cell surface expression of beta-2 microglobulin begins at early blastocyst in the pig (Meziou *et al.*, 1983) although antibodies to the swine MHC do not bind to pre-implantation pig embryos.

Peri-implantation embryos are difficult to obtain and there is little data pertaining to their MHC antigen expression. However, when blastocysts are grown *in vitro* they hatch from the zona pellucida, attach to the substrate, and

appear to mimic some of the early differentiation of the postimplantation period (Sherman, 1975). Trophectoderm-derived tissue in these cultures is negative for cell surface H-2 antigen expression whereas the morphologically undifferentiated tissues (presumably of inner cell mass origin) bind anti-MHC alloantisera to varying degrees (Searle *et al.*, 1976; Heyner, 1973; Sellens, 1977 and Sellens, *et al*, 1978). Co-culture with blastocyst outgrowths does not stimulate long-term, allo-primed cell lines to incorporate ^{3}H-thymidine (Pavia, Stites, and Fraser, 1983).

Teratocarcinomas, tumors presumably arising from this stage of differentiation, have been extensively studied as a model system for antigen expression during development (Jacob, 1977; Gachelin, 1978; Chism, Burton and Warner, 1978; Solter and Knowles, 1979; Martin, Grabel, and Rosen, 1980 and Hamilton, 1983). Undifferentiated stem cells of the tumor are negative for cell surface H-2 (Artzt and Jacob, 1974; Forman and Vitetta, 1975; Vitetta, *et al.*, 1975; Vitetta, *et al.*, 1977; Morello, *et al.*, 1978; Trowsdale, *et al.*, 1980; Knowles, *et al.*, 1980; and Gmur, Solter, and Knowles, 1980) and have virtually undetectable levels of H-2 messenger RNA (Croce, *et al.*, 1981; Morello, *et al.*, 1982; Baldacci, *et al.*; 1983, Kawata, *et al.*; 1984; and Rosenthal, *et al.*, 1984), although beta$_2$ microglobulin is readily detectable. Stem cells can be induced to differentiate (Martin and Evans, 1975 and Strickland and Mahdavi, 1978) and acquire cell surface markers typical of primative endoderm. A teratocarcinoma characteristic of endoderm expresses low levels of H-2 (Searls and Edidin, 1982). Indeed, after induction of differentiation in teratocarcinomas, cell surface H-2 appears, and levels of class I (as well as beta$_2$ microglobulin) message increase dramatically. Teratocarcinoma cells can generate transplantation immunity (Chism, Burton and Warner, 1978 and Johnson, L. L. *et al.*, 1983) and cytotoxic killers (Simmler, Avner, and Levy, 1982) whose specificity is linked to antigen(s) within the MHC. Cell lines established from mouse blastocyts exhibit variable levels of class I determinants (Ostrand-Rosenberg, *et al.*, 1977).

Post-implantation murine embryos have been examined for H-2 antigen expression from day 6 onwards (Johnson, M. H. 1975; Billington and Jenkinson, 1975; and Chatterjee-Hasrouni, Montgomery, and Lala, 1983). Simmons and Russell (1962) first demonstrated the immunological distinctions between extra-embyronic and the embryonic portions of the conceptus. Ectoplacental cone tissue from 7.5 day embryos survived and

proliferated in ectopic allogeneic transfer to hyperimmunized hosts, but embryonic tissues of the same gestational age were promptly rejected. Subsequent studies using transplantation to congenic hosts (Searle, *et al.*, 1974 and Patthey and Edidin, 1973), the inability of ectoplacental cone to generate anti-MHC cytotoxic killers (Pavia, Stites, and Fraser, 1981 and Searle, Jenkinson, and Johnson, 1975), the sensitivity of such cells to specific cytotoxic lymphocytes (Vandeputte and Sobis, 1972 and Jenkinson and Billington, 1974a) and direct binding assays using cells cultured from the embryo (Searle, *et al.*, 1976; Hakansson, *et al.*, 1975 and Sellens, Jenkinson, and Billington, 1978) have confirmed the absence of class I antigens on at least the giant cells of the trophoblast in the early post-implantation period. The expression of these antigens on the proliferating diploid core of ectoplacental trophoblast (which later gives rise to differentiated cells of the placenta) is not clear (Jenkinson and Billington, 1977; Johnson, 1975 and Bell and Billington, 1983), although the small round cells persisting in cultures of ectoplacental cone, presumably the diploid core cells, are H-2 positive (Sellens, Jenkinson, and Billington, 1978). Drake and colleagues (Drake, *et al.*, 1987) have recently shown that short term culture of cells derived from 7.5 day ectoplacental cone are negative for class I and class II antigens. Class I antigens (but not class II antigens) however, can be induced by addition of IFN containing supernantant to the trophoblast cultures. Expression in the embryo itself is difficult to evaluate due to the genetic complexity of the mating and/or immunization pairs and the antisera used, but studies have generally shown the presence of some sort of alloantigen on embryonic cells (Billington and Jenkinson, 1975). Searle and colleagues (Searle, *et al.*, 1974) have demonstrated specific binding of congenically raised anti-H-2 sera to the endoderm of the 7.5 day embryonic sac. Ozato, Wan and Orrisen (1985) have used cocktails of monoclonal anti-class I antibodies and sensitive radioimmunoassay to address this problem. They conclude that class I antigens are absent from cell suspensions of the embryonic portion of 6, 7, and 8 day conceptuses. In addition, these investigators could find no hybridization of a cDNA probe specific for the second external domain of the L^d antigen to total cellular RNA extracted from these early post-implantation stages. Interferons, molecules known to augment class I expression (Rosa and Fellous, 1984), accelerate MHC expression in the embryo by one day; interferon is found in progressively increasing amounts in murine placenta

(Fowler, Reed, and Giron, 1980). Rossant and Croy (1987) have recently reported that whole 7.5 day conceptuses contain very low levels of mRNA that hybridized to a cDNA probe from a highly conserved region of the class I gene. Morello*et al.*, (1985) used exquisitely sensitive S1 nuclease protection assays and found that message for class I heavy chain was barely detectable in blastocysts, 7.5 and 8.5 day embryos. Increasing levels of specific message began to appear in day 9 embryonic and extraembryonic tissue.

Kirkwood and Billington (1981) and Billington and Bell (1983) have shown that class I antigens are first expressed in the day 9-10 embryo, but that this expression is strain dependent, and is found to varying degrees in different parts of the body. Melnick and colleagues (1982) have confirmed such spatial variation of H-2 expression in the 12 day embryo oral plate. Indirect immunofluorescent labeling localizes $H\text{-}2^k$ haplotype determinants to basement membranes and not apical surfaces. Ozato and colleaques (Ozato, Wan, and Orrison, 1985) continued their investigation throughout gestation and found that monoclonal antibodies to the less polymorphic regions of class I antigens first bound to day 9 embryonic tissue, whereas monoclonal antibodies to the more polymorphic determinants first reacted with day 10 embryos. Hybridization of their cDNA probe was first detected in day 9 mRNA extracts.

The expression of class I antigens in the later embryo is more germane to the formation of the fetal immune system than immunoregulation at the maternal-fetal interface, since by day 10 of murine gestation hemopoiesis is beginning in the fetal liver, and the definitive placenta has already been formed (Rugh, 1968, and Begley, Firth and Holt, 1980). Therefore, the expression of allogeneic antigens in mid-and late gestation placenta and fetal membranes becomes paramount to immunoregulatory events. Murine class I antigens are expressed on some populations of placental cells as evidenced by the ability of placenta to specifically absorb anti-class I antibodies from the circulation of pregnant mice (Wegmann, Singh, and Carlson, 1979; Wegmann, *et al.*, 1979; Bell and Billington, 1983c and Adeniyi-Jones and Ozato, 1987). The binding of anti-class I antibodies has been visualized in placenta with autoradiography on tissue sections. There is some question however, as to which region of the placenta contains the H-2 positive cells. Singh, *et al.* (1983) have shown low levels of binding of anti-H-2 antibodies *in vivo* in the spongiotrophoblast, the outer areas of the placenta in direct

contact with the maternal tissues of the uterus. Strong specific immunoperoxidase staining of spongiotrophoblast but not labyrinthine trophoblast in cryostat sections of mature rat placenta with anti-class I antibodies was observed by Billington and Burrows (1986) and Gill's group (Ho, *et al.*, 1987). Chatterjee-Hasrouni and Lala (1982) have localized the antigens to the labyrinthine trophoblast, the more internal areas that first directly encounter the maternal circulation, although recent studies showed the binding of ^{125}I-monoclonal antibody in the spongiotrophoblast as well (Colavincenzo and Lala, 1985). The discrepancy in these results could be due to technical differences (Rossant, 1984), but is accentuated by the fact that the groups used the same monoclonal antibodies for their respective studies. Recently, Adeniyi-Jones and Ozato (1987), using autoradiography of paraffin-embedded conceptuses, found that while most radiolabeled anti-class I antibody localized in the peri-placental yolk sac, the spongiotrophoblast, and to a lesser extent, the labyrinthine trophoblast were also labeled. *In vitro* binding assays show a population of placental cells that possess surface H-2 (Sellens, Jenkinson, and Billington, 1978 and Chatterjee-Hasrouni and Lala, 1979). Jenkinson and Owen (1980) have localized this reactivity to cells of spongiotrophoblast origin. However, Tanaka and colleagues (1983) could not detect cell surface H-2 on trophoblast cell lines using FACS analysis and monoclonal antibodies. In addition, these cells synthesized very small amounts of MHC-specific mRNA (2% of that detected in spleen cell messenger preparations) and reduced levels of beta$_2$ microglobulin mRNA (14% of spleen levels). Mid-and late gestation placental cells can generate cells that react in the mixed lymphocyte reaction with specificity for H-2 determinants (Pavia, Stites, and Fraser, 1981 and Toder, *et al.*, 1982). Spongiotrophoblast cells are superior targets for secondary anti-paternal CTL in these cultures (Smith, 1983a). Spongiotrophoblast cells cannot, however, induce primary CTL, whereas cell cultures derived from the labyrinthine region can induce anti-paternal cytotoxic cell formation *in vitro*, presumably due to contamination of the labyrinthine region by fetal antigen presenting cells (Smith, 1983b). Zuckermann and Head (1986) have recently reported that 20-40% of the cells from short term trophoblast cultures bear low levels of paternal class I antigens as detected by antibody-mediated cytotoxicity and FACS analysis. These authors have also have recently reported that although cultures of day 14-15 placental cells (spongiotrophoblast by morphological and histological

criteria) bind anti-H-2 sera and monoclonal antibodies or compete against tumor targets for binding to NK cells, they were not susceptible to lysis by NK or alloreactive killers (Zuckermann and Head, 1985 a, b). Yolk sac epithelium of mature placenta does not bind anti-MHC antisera (Parr, Blanden, and Tulsi, 1980) although cell cultures from mid-gestation yolk sac or amnion are targets for cytotoxic lymphocytes raised against alloantigens (Jenkinson and Billington, 1974 and Billington and Jenkinson, 1975,). Avery and Hunt (1967, 1968) showed rejection of yolk sac grafts if the inner (fetal) side faced downward; outer (maternal) side-down pieces did not vascularize and engraft.

Distribution of class I within the human placenta is much better described. An early report of HLA antigens present in cultures derived from term placenta (Loke, Joysey, and Borland, 1971) could not be substantiated using absorption assays with freshly isolated trophoblast membrane (Goodfellow, *et al.*, 1976). Fikrig and colleagues (1967) could not stimulate maternal leukocytes to proliferate with trypsinized trophoblast cells or membranes, but Taylor and Hancock (1975) showed that cell cultures of trypsinized placenta can be killed by maternal lymphocytes. Davies and coworkers (1982) raised both humoral and cellular immunity against trophoblast vesicles. The vesicles resisted lysis with anti-HLA or anti-beta$_2$ microglobulin antibody plus complement, but were susceptible to antisera raised against placental alkaline phosphatase. Indirect immunofluorescence and immunoperoxidase, or mixed hemadsorption studies of tissue sections or cell suspensions using alloantisera or monoclonal antibodies have failed to detect HLA antigens or beta$_2$ microglobulin on villous trophoblast (Faulk and Temple, 1976; Faulk and Johnson, 1975; Faulk, Sanderson and Temple, 1977; Sundqvist, Bergstrom, and Hakansson, 1977; Sunderland, *et al.*, 1981a, and Galbraith, *et al.*, 1981) or amniotic epithelium (Adinolfi, *et al.*, 1982 and Hsi, Yeh, and Faulk, 1982) throughout gestation. A recent report suggests that the antigens may be modulated off the surface by binding circulating maternal anti-paternal MHC antibodies (Underwood, *et al.*, 1985). Trophoblast cells isolated from term placenta are surface HLA negative and contain only trace amounts of class I specific mRNA (Kawata, Parnes, and Herzenberg, 1984). However, cell suspensions of cytotrophoblast *in situ* (Montegomery and Lala, 1983), the nonvillous cytotrophoblast (Sunderland, Redman, and Stirrat, 1981b; Redman, *et al.*, 1984; Hsi, Yeh, and Faulk, 1984; Wells, Hsi, and Faulk, 1984; and Loke and Butterworth, 1987) and proliferating cells of

hydatidiform mole (Sunderland, Redman, and Stirrat, 1985) all react positively with monoclonal antibody to the framework or monomerphic regions of HLA antigens. Curiously, none of these studies was able to demonstrate binding of monoclonal antibodies specific for the appropriate allodeterminants of HLA. This argues for either the expression of some incomplete class I molecules, perhaps a necessary structural component of the cell surface (Gill, 1984) or the presence of an as yet unidentified class I determinant characteristic of some population(s) of placental trophoblast (Risk and Johnson, 1986). Power and colleagues (Power, *et al.*, 1983) have evaluated data from family studies and identified antibodies in pregnancy serum with activity against paternal lymphocytes that is linked to MHC, but is not directed against any known HLA specificities. Rabbit immunizations using villous trophoblast membranes produce an antisera cross reactive with trophoblast and lymphocytes, and the antigen recognized is called TLX (trophoblast-lymphocyte cross reactive) (Faulk, *et al.*, 1978; McIntyre and Faulk, 1982; and McIntyre, *et al.*, 1983). This antigen is polymorphic (McIntyre and Faulk, 1982) and may be an unusual form of class I gene product (Faulk and McIntyre, 1983 and Risk and Johnson, 1986).

The evidence for the existence of an unusual class I antigen, Pa (pregnancy-associated), in the rat conceptus is strong. Certain semiallogeneic pregnancies induce a variably titered anti-class I antibody whose reactivity pattern does not correspond to any of the known MHC haplotypes or recombinants (Smith, Sternlicht, and Butcher, 1982; Smith, Margolias, and Sternlicht, 1982; Ghani, *et al.*, 1984; and Ghani, Kunz, and Gill, 1984). The antisera are not complex, probably representing only a few specificities (Smith, *et al.*, 1987) and immunoprecipitate a protein with the biochemical characteristics of a class I molecule (Macpherson, *et al.*, 1987). Expression of such a class I molecule could be important during fetal development. Indeed, Ho, *et al.*, (1987) localized the Pa antigen to the spongiotrophoblast region in the placenta, and the epidermis, hair follicles, spleen, thymic medulla, bronchial and intestinal epithelium, hepatic Kupffer cells, endocardium, endothelium of blood vessels, renal tubular cells and glomeruli and renal pelvis and uterter of fetal and adult tissues. RT1.A^a, a traditional class I rat antigen, had a identical distribution pattern as assessed by indirect immunoperoxidase staining. In addition, Gill's group (1987) has found restriction fragment length polymorphism in the binding of a class I probe (the

repetitive element of pAG64C (Scott, Westphal, and Rigby, 1983)) to genomic DNA of rats heterogygous at the *grc* locus which controls early development (Gill, Siew, and Kunz, 1983). Also, it is worth noting that Wegmann's and Lala's studies of H-2 expression in murine placenta used monoclonal antibodies broadly reactive across many MHC haplotypes, and in some cases heteroclitic on strains unrelated to the immunizing cell (Oi, *et al.*, 1978 and Ozato, Mayer, and Sachs, 1980). Indeed, recent analyses of anti-class I monoclonal antibody banks have shown extensive cross reactions between the classical H-2 antigens, the K, D, and L molecules, and proteins whose structural genes localize to the Qa/Tla region of the mouse MHC (Figueroa, *et al.*, 1983; Cook, *et al.*, 1983 and Sharrow, Flahery, and Sachs, 1984). Thus, it is possible but not yet shown, that the unusual class I antigen detected in rodent and human placentas is a product of the Qa/Tla region.

B) Class II. It is generally agreed that class II antigens are not expressed on trophoblast-derived tissues throughout gestation in the mouse (Jenkinson and Searle, 1979; Raghupathy, *et al.*, 1981 and Chatterjee-Hasrouni and Lala, 1981), rats (Gill, *et al.*, 1987) and humans (Goodfellow, *et al.*, 1976; Faulk and Temple, 1976; Sunderland, *et al.*, 1981a and Galbraith, *et al.*, 1981). Brami and colleagues (Brami, *et al.*, 1983) have shown binding of an anti-class II monoclonal antibody to typsinized cultured human trophoblast cells, but the conditions used could have favored artifactual results (Redman, 1983). HLA-DR positive cells can be found within the stroma of the placental villi and amnio-chorion, but these cells are probably fetal dendritic cells or macrophages, and not exposed to the maternal circulation (Sutton, Mason, and Redman, 1983; Bulmer and Sunderland, 1984; Bulmer and Johnson, 1984; and Loke and Butterworth, 1987). Hunt, King, and Wood (1984) have separated human placenta into several sub-populations of cells and shown that HLA-DR is present in a major fraction which is also enriched for fetal macrophages. Class II expression begins to appear in the hemopoietic tissues of the murine embryo at day 11 and increases throughout gestation (Delovitch, Press, and McDevitt, 1978); these antigens are not found in other compartments of the developing mouse. Class II antigens are expressed in some maternal tissues that surround the feto-placental unit (Jenkinson and Searle, 1979; Searle, Bell, and Billington, 1983; Bulmer and Sunderland, 1984 and Bulmer and Johnson, 1984) and macrophage-like cells within the

endometrium (Johnson and Bulmer, 1984; Bulmer and Johnson, 1985; and Head, 1987). The distribution of these Ia-positive cells may have some importance in the generation of an immune response toward the fetus, since an antigen must be recognized by immunocompetent cells in association with an Ia molecule in order generate a response. Indeed, cells isolated from trypsinization of late gestation murine decidua, deciduoma or placenta present soluble antigen to primed T-cells and cause antigen specific, MHC-restricted proliferation (Elcock and Searle, 1985). This is especially important in light of observations that the Ia-positive cells scattered throughout the endometrium are absent from the immediate vicinity of the implanting blastocyst (Head, 1987). These cells gradually reappear as gestation proceeds.

3. Non-MHC histocompatibility antigen expression.

Preimplantation embryos react with antisera raised across both H-2 and non H-2 genetic differences (Olds, 1968; James, 1969; Heyner, Brinster and Palm, 1969; Palm, Heyner, and Brinster, 1971; Hakansson and Sundqvist, 1975; Muggleton-Harris and Johnson, 1976; Searle,*et al.*, 1976; Billington, *et al.*, 1977 and Parr and Moore, 1977). Since, as we have previously discussed, H-2 specificities are not expressed in cleavage embryos, this reactivity is presumably due to cell surface expression of the so-called minor histocompatibility antigens responsible for weak graft rejection (Snell, Dausset and Nathenson, 1976). Some investigators have used complex antisera on appropriate H-2 compatible strains (Muggleton-Harris and Johnson, 1976 and Billington, *et al.*, 1977) or antisera raised across non-H-2 differences only (Krco and Goldberg, 1976 and Heyner, Hunziker, and Zink, 1980) to show that pre-implantation embryos express minor histocompatibility antigens. Caution must be exercised in drawing these conclusions since many alloantisera have been shown to be contaminated with reactivity against viral antigens (Klein, 1975) whose expression at the embryonic cell surface has yet to be investigated. Allogeneic transplantation to ectopic sites results in failure of further development in the cleavage-stage embryo (Fawcett, 1950; Kirby, 1960; Billington, 1965; Simmons and Russell, 1965, 1966; Kirby, Billington, and James, 1966 and Hunt and Avery, 1976) in either naive mothers or mice presensitized to paternal alloantigens, although continued cell division can produce teratocarcinoma-like masses. Transplanted

blastocysts can survive and develop to some extent (Kirby, 1960; Kirby, 1963a, b, 1969; Billington, 1965; Kirby, Billington, and James, 1966; Avery and Hunt, 1969; James, Acierto, and Murphy, 1972; and James and Yoshida, 1972a, b; Vandeputte and Sobis, 1972; Searle, *et al.*, 1974; Hunt and Avery, 1976, and Hunt, King, and Wood, 1984), although the development of transplanted control syngeneic blastocysts is parallel to that of the allogeneic ones. It is important to note that proper differentiation of the ICM is dependent on the presence of the trophoblast, and vice versa (Gardner, 1975). Thus, evaluation of the subsequent outgrowths is difficult. Both the inner cell mass and trophoblast of blastocyst outgrowth cultures bind non-H-2 antibodies (Searle, *et al.*, 1974; Muggleton-Harris and Johnson, 1976; Searle, *et al.*, 1976; Carter, 1976; Billington, *et al.*, 1977 and Sellens, 1977). Trophoblast of 7.5 day embryos appears to lack non-H-2 transplantation antigens (Billington, 1965; Kirby, Billington, and James, 1966; Simmons and Russell, 1966; Clarke, 1969; Vandeputte and Sobis, 1972; Jenkinson and Billington, 1974; Searle, *et al.*, 1974; Searle, *et al.*, 1976 and Billington, *et al.*, 1977) although outgrowth cultures of egg cylinder stage ectoplacental cone bind anti-MHC and non-MHC sera (Sellens, Jenkinson, and Billington, 1978) and the antigens persist in the embryonic sac. Croy, Rossant and Clarke (1984) have demonstrated that although lymphocytes infiltrate the graft bed of ectoplacental cone transplanted to the kidney, cytotoxic cells are only recovered from xenogeneic but not allogeneic grafts. One report that histocompatibility antigens are revealed in ectoplacental cone following neuraminidase digestion (Currie, van Doorninck, and Bagshawe, 1968) has not been confirmed and is probably a result of artifacts generated by the treatment (Billington and Jenkinson, 1975 and Simmons, *et al.*, 1971). Hulka and Mohr (1968) showed that a second graft of ectoplacental cone to the kidney was rejected in an accelerated manner but they failed to include syngeneic grafts as controls.

Studies on later gestational trophoblast are impossible to interpret due to the histologic complexity of the placenta and/or the lack of appropriate reagents. Rossant and Croy (1985) have recently emphasized this point by showing that most methods of isolating placental cells actually enrich for maternal cells. The development of monoclonal reagents directed against specific epitopes should greatly aid this area of investigation.

4. Expression of onco-fetal antigens (OFA).

Extant immunity to teratocarcinoma can reduce litter sizes in subsequent pregnancies (Breyere and Sprenger, 1969 and Hamilton, *et al.*, 1979). Preimmunization with an immunogenic fibrosarcoma (but not other nonimmunogenic tumors) causes mid-gestational abortion in mice (Tartakovsky, 1987). Many attempts to raise embryo-specific serologic reagents have produced antisera or monoclonal antibodies that cross react with tumors of various origins. The converse is also true (Solter and Knowles, 1979; Hamilton, 1983; and Allison, 1975, Stern, 1984). The distribution of these antigens during preimplantation mouse development varies considerably and biochemical characterization has proven quite difficult even given the availability in some cases of monoclonal antibodies. This difficulty is undoubtedly related to the fact that most monoclonal antibodies to these developmentally regulated antigens recognize carbohydrate determinants on high molecular weight glycolipids and glycoproteins (Stern, 1984 and Feizi, 1985). The saccharide nature of the epitopes recognized could also explain the reactivity of many anti-OFA monoclonal antibodies to "unrelated" adult tissues such as brain and alimentry tract. Thus it appears that stage specific differences in embryonic cell surface molecules ultimately reflect glycosylation differences during development. It is worth noting here that, in the broadest sense, the activity of lectins (carbohydrate binding proteins originally isolated from plants) mimics some growth promoting function in cells (Feizi, 1985), and that teratocarcinoma cells express endogenous lectin-like molecules on their surfaces (Martin, Grabel, and Rosen, 1980). Perhaps the expression of different terminal oligosaccharides at the cell surface is a mechanism by which differential cell growth is maintained. Indeed, one of the antibodies present in syngeneic, hyperimmune antiserum raised against F9 (a murine teratocarcinoma) appears to recognize developmentally regulated structures on a large molecular weight surface glycoprotein (Maramatsu, *et al.*, 1979; Buc-Caron and Dupouey, 1980; and McCormick, *et al.*, 1982). The antibody binds to 8-cell embryos, morulae, and the ICM of the blastocyst. Trophectoderm is negative (Solter and Knowles, 1979). SSEA-1 (a carbohydrate epitope defined by a monoclonal anti-F9 antibody) has a similar distribution pattern (Solter and Knowles, 1978). It was previously thought that the F9 antigen was the wild type product of the T-locus (Artzt, Bennett, and

Jacob, 1974; Kemler, *et al.*, 1976; Marticorena, Artzt, and Bennett, 1978 and Morello, *et al.*, 1980), a region of murine chromosome 17 centromeric to H-2 that regulates early embryonic development (Bennett, 1975). At least some of the T-locus genes however, appear to regulate/encode certain glycosyl transferases (Cheng and Bennett, 1980), and so this association with the F9 determinant probably reflects glycosylation patterns (Stern, 1984).

Not all antibodies to OFA recognize carbohydrate determinants. LDH-X, the isozyme of lactate dehydrogenase characteristic of sperm, is expressed on preimplantation embryos (Bene and Goldberg, 1974). The placental form of alkaline phosphatase is a major cell surface protein of mid- and late gestation human trophoblast, is highly polymorphic and is a major specificity of heteroantisera (Kantor, *et al.*, 1981) and monoclonal antibodies (McLaughlin, *et al.*, 1982 and Travers and Bodmer, 1984) to human trophoblast membranes. Indeed, placental alkaline phosphatase is a marker for syncytial trophoblast (Johnson, *et al.*, 1987). Nevertheless, it remains a mystery why the placental form is the only polymorphic isozyme, and why maternal alloimmunity to the paternally-inherited allele is never observed (Johnson, P. M., 1984). The cell surface transferrin receptor is a prominent protein of villous syncticotrophoblast, but absent from non-villous cytotrophoblast (Hsi, Yeh, and Faulk, 1982; Galbraith, Galbraith, and Faulk, 1980 and Johnson and Molloy, 1983). Transferrin is the major iron-transport protein of the serum, and is essential for cellular growth and proliferation. All rapidly dividing cells have large amounts of transferrin receptor on their surfaces (Newman, *et al.*, 1982). Aside from its availability as a cell surface antigen, the transferrin receptor could be important in sequestering essential iron away from placental villous spaces, thus establishing a competition between maternal blood lymphocytes and fetal placental tissue (Johnson, P. M., 1984). Wiley (1974) has shown binding of an anti-human chorionic gonadotropin (hCG) antiserum to cleavage stage embryos, and anti-hCG antibodies can destroy trophoblast cell cultures in the presence of complement (Currie, 1967 and Morisada, Yamaguchi, and Iizuka, 1976). Antisera to F9, a murine teratocarcinoma, precipitates a protein which contains two chains whose molecular weights are similar to those of the class I molecules (Vitetta, *et.al.*, 1975). The light chain, however, does not react with an anti-beta-2 microglobulin serum (Vitetta, *et al.*, 1977 and Dubois, *et al.*, 1976). Monoclonal antibodies which react with OFA have been generated using trophoblast cells as the immunogen but their

fine specificity has not been determined (Travers and Bodmer, 1984; Johnson, *et al.*, 1981 and Lipinski, *et al.*, 1981, Sunderland, Redman and Stirrat, 1981c and Loke and Day, 1984).

The distribution of OFA in post-implantation embryos is not well studied. A monoclonal antibody to the Forssman antigen (Stern, *et al.*; 1978, and Willison and Stern, 1978) first appears on trophectoderm of late blastocysts, but is confined to ICM derivatives thereafter (Stinnakre, *et al.*, 1981). SSEA-1 is similarly absent from trophectoderm of 6, 7, 8, and 10 day conceptuses and the outer layer of F9 embryoid bodies but present in the embryonic regions of the developing conceptus and the undifferentiated "core" cells of F9 (Fox, *et al.*, 1981). Expression of OFA in the definitive placenta has not been studied.

Recent advances in tumor biology arising from the use of molecular genetic tools have provided a different approach to the investigation of OFA in pregnancy tissue. cDNA probes from a wide variety of *v-* and *c-oncogenes* are increasingly available (Bishop, 1983 and Varmus, 1984) and some have been used to screen RNA of human and mouse placenta. Elevated expression of *c-myc* is seen in first trimester human placenta and then declines. *In-situ* hybridization localized the abundant message to the cytotrophoblastic shell overlying the chorionic villi. The *myc* gene encodes a protein found in the nuclei of transformed cells and is thought to control proliferation via direct interactions with the DNA (Pfeifer-Ohlsson, *et al.*, 1984). *C-sis*, the gene which encodes the B chain of platelet-derived growth factor (Doolittle, *et al.*, 1983 and Waterfield, *et al.*, 1983) is also expressed at high levels in the cytotrophoblastic shell of the early human conceptus (Goustin, *et al.*, 1985). The mouse fetus, placenta and associated membranes show stage specific expression of the *c-fos*, *c-abl*, and *c-fms* gene transcripts (Muller, *et al.*, 1982; Muller, Verma, and Adamson, 1983 and Muller, *et al.*, 1983). *c-fos* message is found at levels approaching those observed in transformed cells in midgestation mouse and human cytotrophoblast and late gestation extraembryonic membranes. *c-fms* expression is high in placenta but not fetal membranes. *c-abl* message is easily seen in midgestation embryonic tissues but is low during early and late pregnancy and not significant in extraembryonic tissues throughout gestation. *c-ras* is constitutively expressed at high levels in all tissues of the conceptus during pregnancy. *c-fos* encodes a protein that is located in the nucleus; *c-abl*, *c-fms* and *c-ras* gene products are plasma membrane proteins that are probably equivalent to growth factor

receptors (Land, Parada, and Weinberg, 1983; Heldin and Westermark, 1984; and Rettenmier, *et al.*, 1985). Wahlstrom and colleagues (1984) found cross reactions to retrovirus structural proteins in their monoclonal antibodies generated against syncytiotrophoblast. Clarke, Gelmand and Reitz (1984) have shown homology between the HTLV envelope protein and the HLA-B gene at the DNA level.

Some xenogeneic and syngeneic immunizations using mouse gestational tissue have produced antibodies that react with embryonic and not tumor or adult tissues (Bagshaw and Lawler, 1975; Wiley, 1979; Solter and Knowles, 1979 and Johnson and Calarco, 1980). However the antisera may not have been tested against a panel of sufficient diversity to reveal such cross reactivities.

C. Does the Mother Respond Immunologically to the Conceptus?

Alloantibody and anti-oncofetal antibody responses to mammalian pregnancy have been demonstrated in many species (Bernard, 1977; Hamilton, 1983; Billington and Bell, 1983; Bell, Billington, and Smith, 1983 and Gill, 1985). In most cases, however, antibody is not detected until late during or after the second pregnancy. These findings raise doubts about the nature of the antigenic stimulus since there is a large leakage of fetal blood into the maternal circulation at parturition, at least in humans (Gill, 1977). Nevertheless, trophoblast cells can be found in maternal blood (Herzenberg, *et al.*, 1979 and Covone, *et al.*, 1984) and spleen (Liegneois, *et al.*, 1981 and Philip, Ayraud, and Masseyeff, 1982) during the first pregnancy. It is therefore quite possible that primiparous mothers can be sensitized by an antigen dose which is insufficient to produce circulating antibody, and that the resultant memory cells can be triggered by subsequent pregnancies to yield highly titered antibody responses. Over 90% of primiparous mares develop high titer anti-paternal alloantibody (Antczak, Miller, and Remick, 1984). However, a humoral response to pregnancy is not universal, even if the same antigenic stimulus is supplied in succeeding gestations. About 15-25% of primiparous (van Rood, van Leeuwen, and Eernisse, 1959; Overweg and Engelfriet, 1969; Ahrons, 1970; van der Werf, 1971; Doughty and Gelsthorpe, 1974; Gill and Repetti, 1979 and Gill, 1985) and 40-60% of multiparous women (Doughty

and Gelsthorpe, 1974, 1976 ; Gill and Repetti, 1979; Terasaki, *et al.*, 1970; Carbonara, *et al.*, 1974; Winchester, *et al.*, 1975 and Ferrone, *et al.*, 1976) produce anti class I and class II HLA agglutinating and cytotoxic antibodies. Anti-HLA (Faulk, *et al.*, 1974 and Jeannet, *et al.*, 1977) and anti H-2 (Bell and Billington, 1980, 1981 and 1983a) activity can be detected in placental eluates.

Anti-H-2 antibodies are found only in a few mating combinations in mice (Billington and Bell, 1983; Bell, Billington, and Smith, 1983; Taylor, 1973; Roe and Bell, 1982 and Bell, 1984) and rats (Smith, Sternlicht, and Butcher, 1982; Smith, Margolias, and Sternlicht, 1982; Ghani, *et al.*, 1984 and Ghani, Kunz, and Gill, 1984) and the responder/non-responder status is MHC-linked. The antibodies produced are of limited specificity and thus a subset of the broadly cross reactive sera produced by spleen cell or skin graft immunization in the same strain combinations. This observation suggests a limited display of MHC epitopes on gestational tissue. Indeed, responder mice preimmunized to paternal spleen cells produce the broad spectrum antibody characteristic of conventional alloimmunization during pregnancy (Roe and Bell, 1982). Such broadly reactive antibodies can also be found in non-responder primigravida (who cannot be primed for antibody production by successive pregnancy alone). In addition, multiparous females of responder strains will produce an anamnestic antibody response of limited scope if later challenged with splenocytes. Intraperitoneal immunization using tissues of gestational origin reveal that fetal cells induce the conventional alloantibody whereas placental cells produce the restricted response (Bell and Billington, 1983a).

Priming of T-cell responses as a consequence of pregnancy may be difficult to demonstrate due to the complexity of the assays employed. Failure to detect a response can be due to a lack of specific cells or secreted factors needed for the expansion or maintenance of the T-cells in the assay rather than a lack of appropriate activation *in vivo*. Alternately, various mitogens present in the culture media can activate cells via mechanisms that are unrelated to their stimulation by specific antigen, thus giving rise to false positives. The literature is crowded with reports that claim the range of cell mediated immunity from the lack of a detectable response through active lymphokine production, mixed lymphocyte reactivity (MLR), delayed-type hypersensitivity (DTH), cytotoxicity (CTL) and suppression (Bernard 19[illegible], Gill and Repetti, 1979; Gill, 1985 and Jacoby, Olding, and Oldstone, 1984).

The lymphocytes of primiparous females are not primed to paternal antigens since they do not react with secondary response kinetics to paternal strain cells in an MLR (Hamilton and Hellstrom, 1977; Pavia and Stites, 1979; and Smith, 1981), CTL (Wegmann, *et al.*, 1979b and Pavia and Stites, 1979), adoptive DTH (Maroni and Parrot, 1973; Harrison, 1976a and O'Hearn and Hilgard, 1981). There is no *in vivo* immune clearance by primiparous mothers of labeled tumor cells bearing paternal H-2 antigens (Wegmann, *et al.*, 1979b.) Hellstrom and Hellstrom (1975) detected anti-tumor activity in primigravida spleen, but this could be due to the activity of natural killer (NK) cells. While some investigators detect no change in cell-mediated reactivity to paternal alloantigens as a consequence of multiple pregnancy (Pavia and Stites, 1979; Carr, Stites, and Fudenberg, 1974; and Sargent, Redman, and Stirrat, 1982), Smith, *et al* (1978) obtained intermitent cytotoxicity in primiparous spleen. Yet others have observed hyporesponsiveness (Andresen and Monroe, 1962; Lewis, *et al.*, 1966; Hellstrom, Hellstrom, and Brawn, 1969; Ceppellini, *et al.*, 1971; Fabris, Plantanelli, and Muzzioli, 1977; Gottesman and Stutman, 1980 and Head, 1982), increased reactivity (Maroni and Parrott, 1973; Rocklin, *et al.*, 1973; Tait, d'Apice, and Morris, 1974; Hamilton, Hellstrom, and van Belle, 1976 and Baines, *et al.*, 1976) or evidence for the priming of a T-cell response (increased proliferation in response to paternal antigens), but no increase in CTL activity (Smith, 1981 and Gambel and Ferguson, 1982). Youtananukorn and Matangkasombut (1972) showed that postpartum maternal lymphocytes proliferated in response to placental cells from their own, or a few third-party placentas.

Natural killer (NK) cells are bone marrow-derived null lymphocytes which can kill without previous sensitization (Henney and Gillis, 1983). NK cell activity is present in the pregnant mouse spleen (Chatterjee-Hasrouni, Parhar, and Lala, 1984), but unchanged (Todar, *et al.*, 1983) or slightly depressed (Baines, Pross, and Millar, 1978; Barrett, Rayfield, and Brent, 1982 and Baley and Schacter, 1985) in the mononuclear cells isolated from human blood during pregnancy. Decidual cells have high NK activity during the peri-implantation period (6.5-8.5 days) but this activity becomes negligible by day 10 (Croy, *et al.*, 1985). Indeed, large numbers of decidual lymphocytes (8-10% compared to 2-4% of splenocytes) from day 12-14 decidua bound NK targets, but NK lysis was reduced compared to that obtained with splenocytes (Lala, Kearns, Parhar, 1987). The presence of decidual cells in a 1:1 ratio with

normal splenocytes eliminated the NK activity of these cells, but this suppression is sensitive to low concentration of indomethacin, an inhibitor of prostaglandin synthesis. Prostaglandins are potent immunosuppressive agents (Johnson, *et al.*, 1983). Placental cells compete with YAC-1 cells (the traditional *in vitro* NK target) and thus bear target structures recognized by NK cells, but are not lysed *in situ* (Chatterjee-Hasrouni, Parhar, and Lala, 1984 and Zuckermann and Head, 1985). Placental cell vesicles (Davies, McLaughlin and Sutcliffe, 1982) are susceptible to lysis by NK cells.

D. Can the Placenta Prevent the Passage of Immune Effectors Into the Fetus?

Systemic alloimmunity in the gestating female implies either a local crippling of immune effectors or a barrier between mother and fetus in successful pregnancy. The evidence for suppression of the immune response during pregnancy will be discussed in the following section. Empirical evidence for the existence of a barrier was obtained when the offspring of multiparous, transfused, or grafted females with circulating antibody developed no abnormalities during subsequent pregnancies (Currie, 1969 and Simmons and Russell, 1967). Early experimental attempts to compromise pregnancy by inducing a vigorous alloimmune response failed (Mitchison, 1953; Woodruff, 1958 and Lanman, Dinerstein, and Fikrig, 1962). Swinburne (1970) first proposed the idea that the placenta could act as a specific "sponge", expressing target antigens which bind circulating maternal antibody. The resultant immune complexes were thought to contribute to the fibrinoid deposition often seen in the placenta and thus create a barrier. The rabbit placenta is an efficient antibody filter since anti-MHC antibodies are absent from cord blood but trapped in (and can be eluted from) the placenta if the conceptus bears the target antigen. Offspring whose MHC type differed from that eliciting the circulating antibody had little placentally bound antibody, but high titers in cord and fetal blood (Lanmand and Herod, 1965). Taylor (1973) reported that half the offspring of the litter of hyperimmune, allogeneically mated females contained cytotoxic antibody, whereas antibody was present in all neonates if they were of an irrelevant MHC haplotype. Similar phenomona have been noted in human pregnancy (Doughty and Gelsthorpe, 1976 and Tongio and Mayer, 1975). Dr. Wegmann's laboratory

has extensively studied the immunoabsorbent capacity of the murine placenta. Initially, the group monitored the disappearence of passively administered anti-paternal cytotoxic serum from maternal circulation (Wegmann and Carlson, 1977 and Carlson and Wegmann, 1978) and subsequently showed that target placentas bound ^{125}I-labeled serum-derived antibody (Wegmann, Singh, and Carlson, 1979) or monoclonal anti-MHC antibody (Wegmann, *et al.*, 1979), thus preventing its accumulation in the fetus. The concentration of epitopes within the placenta is low (Wegmann, *et al.*, 1980) but the bound antibody is internalized and degraded within 4-6 hours (Raghupathy, Singh, and Wegmann, 1984) and the capacity of the antigen sponge is regenerated within 48 hours (Raghupathy, *et al.*, 1981).

Billington's group (Billington and Bell, 1983 and Bell and Billington (1983c) has shown that the placenta is not an efficient barrier when confronted with high titers of extant maternal circulating antibody. Both the allogeneic placenta and fetus contained the antibody when maternal anti-paternal serum titers exceeded 1:128. When these mice were subsequently syngeneically mated, the alloantibody bound to placenta only when circulating titers exceeded 1:128, while the fetal serum was positive for placentally-passaged antibody if maternal titers were greater than 1:16. The immunogenic stimulus for antibody production was pregnancy. However, antibodies were never detectable until late in the second gestation. Thus, it is possible that the fetal bleed at parturition provides an antigenic stimulus containing epitopes not present on placenta and therefore not absorbed by it in subsequent pregnancy. Nonetheless, it must be noted that the maternal circulating antibody induced in multiparous responders is of limited specificity compared to that evoked by lymphocyte challenge. The fine specificity of this antibody spilling over into the fetus has not been determined. Adeniyi-Jones and Ozato (1987) have also shown that the immunoabsorbent capacity of the placenta is limited. Target placentas bound more radiolabeled monoclonal anti-class I antibody than control placentas only when 0.4 ng or 2 ng of protein A-purified, ^{125}I labeled ascites was injected into maternal circulation. Larger doses (up to 2 mg) did not discriminate target versus control. Intact antibody accumulated in the fetuses regardless of haplotype.

The trafficking of sensitized cells from mother to fetus is difficult to assess experimentally, and is usually associated with fetal abnormalities. Chimerism of maternal cells has been found in children with severe combined

immunodeficiency syndrome (SCID) (Githens, *et al.*, 1969; O'Reilly, *et al.*, 1973; Pollack, *et al.*, 1980, 1982; Rosenstock, Goldblum, and Sharp, 1981; Geha and Reinherz, 1983 and Flomenberg, *et al.*, 1983). Severe runting has been shown in rats following transfer of hyperimmune cells specific for the alloantigens of the fetus (Beer, Billingham, and Yang, 1972 and Beer and Billingham, 1973). The severity of the disease increases with increasing cell dosage, but no abnormalities were observed during gestation. The possibility that the neonates were attacked by effectors present in the mammary glands was not considered in these studies. Indeed, foster nursing of newborn F1 strain rats by Lewis strain mothers can produce tolerance of Lewis skin grafts in the offspring (Beer, Billingham, and Head, 1975 and Head, Beer, and Billingham, 1977). If the transfer to foster mothers was delayed by a day, the skin grafts showed the accelerated, second set rejection characteristic of previous priming. These experiments could not be repeated (Silvers and Poole, 1975) and allogeneic foster nursing did not compromise neonatal nude mice (Hetherington and Hegan, 1978). Nevertheless, adoptively transferred, radiolabeled lymphocytes can be detected in large numbers (5% of cells obtained) in the milk of lactating rats (Head and Seelig, 1983). Maternal lymph node cells force-fed to neonatal rats can be detected intact in the lumen and walls of small gastric blood and lymphatic vessels (Seelig and Head, 1987), showing that ingested cells can migrate out into the surrounding tissues.

Thus, the transplacental passage of sensitized, activated cells in normal uncomplicated pregnancy has not been established. The ability of the placenta to act as an anatomical barrier to all cellular traffic has been hotly debated, and there is evidence to support all conclusions (Billingham, 1964; Schroder, 1975; Gill, 1977; 1985, Gill, and Repeti, 1979; Loke, 1978; and Chaouat, Kolb, and Wegmann, 1983). Care must be taken in conducting such studies to insure that a) maternal markers are easily distinguishable in the presence of an overwhelming number of fetal cells, b) the artificial labeling of maternal cells (if any) does not upset their normal circulation and homing patterns, c) the label cannot be released from maternal cells as a soluble species that can cross the placenta and label fetal cells *in situ*, and d) the integrity of the placenta is not violated.

E. What is the Role of Immunosuppression in the Maintenance of Pregnancy?

The failure to observe an effective immune response to antigenic stimuli can be attributed to many causes. Included in these are: insufficient dose or inappropriate physical form of antigen, clonal deletion or anergy of the appropriate responding cells, presence of various hormones and other proteins which generally depress immunity, pre-existing circulating antibody which masks the relevant epitope and thus blocks recognition, or the generation of a cell population which suppresses the response (Moller, 1984). All of these possibilities have been investigated as potential aids to the maintenance of semiallogeneic pregnancy.

As we have previously discussed, alloantigens and tissue/stage-specific antigens are expressed in the conceptus, albeit in low amounts or inaccessible locations, especially within the trophoblast. Yet, the mother can and often does mount an immune response as a consequence of pregnancy. Clearly, then, the absence of an immunogen or immunocompetent cells cannot explain the tolerance of her semiallogeneic fetus by the mother.

Pregnancy proteins are capable of generalized suppression of *in vivo* and *in vitro* immunity. A variety of cell mediated responses including MLRs, lymphokine production, and mitogen-induced proliferation are inhibited by gestational hormones, particularly progesterone and hCG (Schroder, 1975; Gill and Repetti, 1979; Jacoby, Olding, and Oldstone, 1984 and Stites and Siiteri, 1985). Alpha-fetal protein (AFP), the fetal equivalent of serum albumin which is produced in the liver, and to a lesser extent by the yolk sac, is a well documented inhibitor of cellular and humoral immunity (Murgita and Tomasi, 1975a, b; Murgita, *et al.*, 1981 and Suzuki and Tomasi, 1980). AFP probably acts by preventing the cell surface expression of Ia antigens (Peck, Murgita, and Wigzell, 1978 and Lu, Changelian, and Unanue, 1984) and/or the generation of suppressor cells (Murgita, *et al.*, 1981; Murgita, *et al.*, 1977 and Toder, Blank and Nebel, 1982). Other proteins, whose concentrations are increased in maternal serum during gestation, can dampen immune responses (Gill and Repetti, 1979). The significance of these effects remains doubtful since in many studies the concentrations of putative inhibitors used far exceeded their maximum gestational serum levels. At physiological

concentrations, the suppression is not observed. In fact, very high concentrations of purified hCG (Caldwell, Stites, and Fudenberg, 1975 and Morse, *et al.*, 1976) or AFP (Charpentier, *et al.*, 1977 and Sheppard, *et al.*, 1977) can augment an MLR. It is important to note that one of the major functions of AFP is to act as a transport protein for serum macromolecules, and thus it is often bound to molecules with known immunosuppressive action, such as progesterone (Murgita, 1976). Nevertheless, these nonspecific immunoregulatory proteins can be quite concentrated at their source and thus exert local immunosuppression (Pavia, *et al.*, 1979). However, most placental proteins can be localized to the villous syncytiotrophoblast by immunocytochemical labeling of paraffin-embeded sections (Loke and Butterworth, 1987). Thus the regulatory substances are, in the main, presumably secreted into maternal circulation and not available in the placental bed (Jacoby, Olding, and Oldstone, 1984 and Clark, *et al.*, 1984b).

We already discussed the presence of anti-paternal antibodies in and around the placenta. Antibodies eluted from murine placenta and administered to a naive recipient allow the growth of a paternal strain (but not third party) tumor (Voisin and Chaouat, 1974). The effect is slight, but presumably due (at least in part) to the masking of the antigens relevant to the stimulation of effective immunity in the host by the blocking antibody (Chaouat, *et al.*, 1979). However, passive transfer of serum from parous mice will not enhance tumor growth in naive recipients (Kaliss and Dagg, 1964), although serum titers may be low if large amounts of antibody are bound to the placenta (Rocklin, Kitzmiller, and Kaye, 1979). Anti-paternal antibodies in parous serum (Hellstrom, Hellstrom, and Brawn, 1969; Jenkins and Hancock, 1972; Robert, Betuel, and Revillard, 1973; Revillard, *et al.*, 1973; Youtananukorn and Matangkasombut, 1973; Gatti, *et al.*; Hellstrom and Hellstrom, 1975, 1975; Pence, Petty, and Rocklin, 1975; Rabson, *et al.*, 1976; Harrison, 1976; Rocklin, *et al.*, 1976; Pavia and Stites, 1979 and Stimson, Strachan, and Shepherd, 1979) or eluted from placenta (Revillard, *et al.*, 1973; Bonneau, *et al.*, 1973; Faulk, *et al.*, 1974; McIntyre and Faulk, 1978, 1979; Chaouat, *et al.*, 1979; Rocklin, Kitzmiller, and Garvoy, 1982; and Stewart, *et al.*, 1984) can specifically block lymphokine release, MLR against paternal lymphocytes, and the generation of cytotoxic killers *in vitro* (Bernard, 1977 and Rocklin, Kitzmiller, and Kaye, 1979). Several pregnancies may be required to produce the blocking antibodies. Often the anti-MHC antibody obtained in response to

pregnancy is of the same specificity but different isotype from that produced in response to intraperitoneal or intravenous allogeneic lymphocyte challenge. Non-complement fixing IgG_1 is preferentially produced when pregnancy provides the stimulus in some (Bell and Billington, 1980, 1981, and 1983a) but not all (Loke, 1978) murine pregnancies. A strong, complement-binding anti-MHC IgG_{2a} antibody response to allogeneic cells can be shifted to an IgG_1 response when placental cell (especially spongiotrophoblast) extracts are included with the immunogen (Duc, et *al.*, 1983 and Gupta, *et al.*, 1983). The isotype of anti-MHC antibody in the rat is also shifted during pregnancy, but in this species IgG_{2a} is predominantly produced. This antibody is also non-complement fixing (Ghani, *et al.*, 1984a). Nevertheless, the relevance of these changes in the nature of the antibody response is questionable in light of the fact that a humoral response is not always present during pregnancy. Indeed, Rodger (1986) has shown that mice made B-cell deficient by continuous treatment with anti-IgM antiserum had normal pregnancies, and long term neonatal survival rates were the same in the treated or control litters. Thus, blocking or modulating antibodies may be considered epiphenomena or back-up mechanisms (Lala, *et al.*, 1983). Evidence for the regulation of immune responses based on anti-idiotype networks is increasing (Eichmann, 1978; Urbain, Wuilmart, and Cazenave, 1981; and Paul and Bona, 1982). Some investigators have found anti-idiotype antibodies during pregnancy in the mouse (Gatti, *et al.*, 1975; Chaouat and Voison, 1979; Suciu-Foca, *et al.*, 1983 and Singal, *et al.*,1984) and the significance of this potential control mechanism is currently being debated (Mowbray and Underwood, 1985).

Placental cells or supernatants of placental cultures can non-specifically suppress cell mediated responses *in vitro* (Barg, *et al.*, 1978, Chaouat, *et al.*, 1980b, Pavia and Stites, 1981, Remacle-Bonnet, Rance, and Depieds, 1983, van Vlasselaer and Vandeputte, 1984, Mayumi, *et al.*, 1985, and Chaouat and Kolb, 1984). Suppression of NK responses by cells enriched for trophoblast cells (Kolb, Chaouat, and Chassoux, 1984) is also observed, although recent studies suggest that much of the suppression in placental cell cultures may be associated with fetal erythrocytes or small non-trophoblast cells (Clark and Chaouat, 1986). Decidual cells can suppress an MLR and the suppression is abrogated by indomethacin, but restored by exogenous prostaglandin (Parhar and Lala, 1985). Decidual cell culture supernatants (Golander, *et al.*, 1981; Kirkwood and Bell, 1981 and Badet , Bell, and Billington, 1983) also suppress

in vitro cell mediated immune reactions. Caution in interpretating these results is advised by recent experiments of Croy and colleagues (1983). They have demonstrated that the non-specific suppression of cytotoxicity achieved by embryonic tissues can be equal to or greater than that shown by trophoblast or decidua. Drake and Rodger (1985) observed suppression of both the alloantigen and mitogen induced proliferative responses in the presence of ectoplacental cone and mid-gestation trophoblast culture supernatants. However, supernatants of embryonic sac, adult lung, liver and myeloma were similarly suppressive.

Cells that suppress cellular and/or humoral anti-paternal responses can be found systemically in the gestating female (Rees, *et al.*, 1975; Hamilton and Hellstrom, 1977; Smith and Powell, 1977; Chaouat, *et al.*, 1979; Suzuki and Tomasi, 1979; Chaouat and Voisin, 1980, 1981; Chaouat , *et al.*, 1980a, b, 1982; Nicklin and Billington, 1982 and Sano, *et al.*, 1984) but the presence or absence of circulating suppressors has no bearing on the outcome of pregnancy (Faulk and McIntyre, 1983; Clark, *et al*, 1984a; and Chaouat and Monnot, 1984). Pavia and Stites (1979) reported an absence of both circulating cytotoxic cells and suppressors. Smith (1981) noted that either helper or suppressor T cells were generated during pregnancy in a strain dependant fashion. Chaouat and colleagues (Chaouat and Kolb, 1984, Chaouat, *et al.*, 1980 and Chaouat and Chaffaux, 1984) confirmed that placental extracts can generate cells from spleen that suppress tumor rejection and adoptive DTH.

Some reviewers have argued that systemic suppression is irrelevant but that local active suppression is critical to fetal viability (Clark, *et al.*, 1984a,b and Chaouat and Monnot, 1984). Clark and colleagues have characterized the suppressor cells found in the lymph nodes draining the uterus and the decidual cells and cells scraped from the walls of the gravid uterus (Clark, *et al.*, 1984a, b, and 1987). Local active non-specific suppression is bimodal and accomplished by two different populations of suppressors, phase A and phase B cells (Clark, *et al.*, 1987). Phase A suppressors apparently arise in response to hormonal changes early in pregnancy. Indeed, these cells can be generated in the uterus with hormone therapy that produces pseudopregnancy, that is, in the absence of an embryo (Clark, McDermott, and Szewczuk, 1980; Clark and McDermott, 1981; Slapsys and Clark, 1982; and Brierley and Clark, 1985). These large, $Thy1^+$, $Lyt\ 2^+$ cells prevent the

generation of CTLs from precursors (pCTL) but do not affect antibody responses and are only present in the uterus during the preimplantation phase (Brierly and Clark, 1987). The kinetics of phase A cell activity in the lymph nodes draining the uterus is similar, but delayed by a day.

Phase B suppressors are small, granulated, non-T, non-B lymphocytes generated only in the presence of viable trophoblast. The suppressors are absent from failed pregnancies such as the resorbing implantation sites of CBA/J x DBA/2J (a mating combination with a high rate of spontaneous abortion) or *mus musculus* x *mus caroli* (a non-viable interspecies murine mating) (Clark, McDermott, and Szewczuk, 1980; Clark, *et al.*, 1983 and Slapsys and Clark, 1982). CTLs can be easily obtained in the absence of suppressors (Croy, Rossant and Clark, 1982 and Clark, *et al.*, 1987). Indeed, *mus caroli* embryos can complete gestation and produce healthy adults if surrounded by *mus musculus* trophoblast before transfer to the *mus musculus* uterus (Rossant and Frels, 1980; Rossant, Mauro, and Croy, 1982 and Rossant, *et al.*, 1983). Irritants or tumors of non-placental origin do not induce the suppression (Nagarkatti and Clark, 1983) but placentally-derived choriocarcinoma recruits the phase B suppressors (Slapsys, Beeson, and Clark, 1984). Like the early phase A suppressors, the phase B cells are not specific for or restricted by the MHC antigens. The suppression inhibits the proliferative response of T-cells to interleukin-2 (Clark, *et al.*, 1985), the development of cytotoxic killers in sponge matrix allografts and the antibody response and can be mediated by a soluble factor (100,000 daltons) (Clark, *et al.*,1984b). Hunt, Manning and Wood (1984) isolated potent suppressors of the T-cell mitogen proliferative response from the uterus late in murine gestation. These cells were identified as macrophages on the basis of adherance to culture dishes and several cell surface markers.

Suppressor cells are the most active T-cell subset isolated from human cord blood (Jacoby, Olding, and Oldstone, 1984). Fetal and neonatal T-cells (but not monocytes or B-cells) suppress maternal lymphocyte proliferation *in vitro* and responses to B-cell mitogens (Olding and Oldstone, 1976 and Unander and Olding, 1981) probably via a soluble factor (Olding, Murgita, and Wigzell, 1977). The suppressor effectors are OKT8+, an unusual phenotype (Jacoby and Oldstone, 1983 and Yachie, *et al.*, 1981), and the inhibition of prostaglandin synthesis abrogates the effectiveness of these cells. Adult lymphocytes are one hundred times more sensitive to inhibition by

prostaglandins than neonatal lymphocytes (Johnson, *et al.*, 1983). This difference in sensitivity of maternal and fetal lymphocytes to prostaglandins (presumably due to a lack of appropriate receptors in the fetal cells) suggests an elegant control of potentially deleterious immune responses at the maternal-fetal interface (Jacoby, Olding, and Oldstone, 1984). Murine neonatal spleen and liver are also a good source of non-specific suppression for T- and B-cell responses (Globerson, Zinkernagel, and Umiel, 1975 and Main and Pierce, 1983). The suppressors are Lyt 1+ T-cells of the suppressor-inducer circuit (Murgita, *et al.*, 1981).

F. Can an Ongoing Immune Response Enhance Fetal Viability?

We have previously discussed the immunologic enhancement of tumor growth in parous females. This phenomenon is probably due to escape from the cell mediated response, the major mechanism for the elimination of tissue grafts (Klein, 1983), via the masking of antigenic determinants by circulating blocking antibodies. Since antibodies (which can block specific cell mediated responses *in vitro*) can be eluted from placental tissue, it is assumed that they play a role in preventing an "allograft rejection" of the fetal-placental unit (Rocklin, Kitzmiller, and Kaye, 1979). Earlier investigators have argued, however, that an ongoing immune response can benefit the fetus by increasing placental size. The magnitude of the decidual reaction is not consistently affected by the degree of genetic disparity between mother and fetus (Hetherington, 1971, 1972 and 1973) but preimmunization with paternal splenocytes can decrease the size of the decidualization of the endometrium (Clarke, 1971; Clarke and Hetherington, 1971 and Hetherington and Humber, 1975). Billington (1964) observed that F_1 murine placentas were larger than those produced by either inbred parental strain and that inbred parental strain embryos transferred to the pseudopregnant uterus of the other strain also had larger placentae. Preimmunization enhanced these effects: females immune to paternal antigens produced larger placentas while mothers tolerant of paternal antigens had both smaller placentas and fetuses (James, 1965 and 1967). Clarke and Kirby (1966) and later Bodmer (1972) theorized that an immune response was necessary or at least beneficial to pregnancy, and could be the driving force behind the maintanance of a high rate of

polymorphism at the MHC. McLaren (1975) concluded that there was little basis for these claims since a) placental size in F_1 hybrids can be increased or decreased, compared to the inbred paternal strains, depending upon the parental strains, b) the increased size that is more common in outcrosses can be attributed to heterosis (i.e. hybrid vigor, the increase in size and performance usually associated with heterozygosity of recessive lethal alleles), and c) changes in placental weight and litter size in immunized versus tolerant mothers were also strain dependant with the differences barely significant at best. Beer and Billingham (1977) reported that local immunity improved reproductive efficiency. Females that were presensitized by paternal strain skin grafted to one horn of the uterus possessed more implantations in the immunized horn than the untreated one. Implantation number of syngeneic embryos was not affected by preimmunization. A careful study in mice, rats, and hamsters showed that maternal systemic preimmunization did affect placental size, albeit slightly and that the increase in immune versus naive or tolerant rats was abrogated if the regional lymph nodes were removed prior to mating (Beer, Scott, and Billingham, 1977). A major criticism of this and other studies was that the size and general health of the mother was not stated. Since graft-versus-host disease is a common problem in the establishment of neonatal tolerance, the presence of such a sub-clinical syndrome could have compromised reproductive performance and lowered placental and fetal size (Hetherington, 1973; Hetherington, Humber, and Clarke, 1976 and Hetherington and Fowler, 1978). Hetherington (1978) found the opposite result, namely that tolerant mice produced larger placentas than naive mothers. He could not support the idea that immunostimulation produced more viable offspring after comparing implantation number and post-implantation mortality in mating regimens involving seven strains (Hetherington and Fowler, 1978), although placental size generally increased with parity (Hetherington, 1972). Breyere and colleagues (Breyere, *et al.*, 1974 and Breyere, 1976) have shown that placental weight first decreases with parity in both syngeneic and allogeneic matings. Beyond three pregnancies, however, the placentae become increasingly larger. In contrast, tumor enhancement is minimal during the first pregnancy, but rises steadily to a maximum at about the fourth pregnancy (Breyere, 1976). Thus the immunological effector mechanisms (if any) may be related but not identical in these situations and pregnancy may evoke more than one type of immunity.

Blakely (1978) added further complexity to these observations by observing that not only placental size but also growth rate varied with strain combination. Thus the different results from other laboratories could be influenced by the gestational age of the placentae and fetuses examined. Sex differences have also been noted: placentae of male embryos are generally larger than those of females embryos, although there is no distortion of sex ratios in the number of neonates or weanlings (Beer and Billingham, 1977, and Blakley). Some investigators have noted that certain mouse (Hull, 1969 and Hamilton and Hellstrom, 1978) and rat (Palm, 1969, 1974) backcrosses produce an excess of heterozygotes with respect to histocompatibility antigens, but the slight effects usually required multiple pregnancies, or were difficult to confirm (Hetherington, 1973 and McLaren, 1975).

Several observations with respect to spontaneous abortion in humans have stimulated a renewed interest in immunostimulation as a mechanism for maintaining fetal viability. Fetal wastage is apparently very common, especially in early early human pregnancy, and only a few of these failures have an obvious genetic basis (Gill, Siew, and Kunz, 1983). The frequency of HLA-sharing (and possibly ABO-sharing) (Gill, 1983 and Gill, *et al*, 1987) in couples suffering from chronic spontaneous abortion but no obvious chromosomal, microbiological, endocrinological, or gynecological abnormalities, was increased when compared to uncomplicated pregnancies (Komlos, *et al.*, 1977; Gerencer, *et al.*, 1979; Faulk and McIntyre, 1981; Beer, *et al.*, 1981; Unander and Olding, 1983; McIntyre, *et al*, 1984 and Gill, 1985). It was further noted that the blocking antibodies which inhibit maternal anti-paternal MLR were absent from the sera of spontaneous aborters (Harris and Lordon, 1976; Bernard, 1977; Stimson, Strachan, and Shepherd, 1979; Rocklin, Kitzmiller, and Garvoy, 1982; Unander and Olding, 1983; McIntyre, *et al.*, 1984; and Gill, 1985) and women suffering from preeclampsia (Kitzmiller, 1977 and Pattillo, 1980). T-cell proliferative responses were unimpaired in most studies, however, Halbrecht and Komlos (1968) and Lauritsen's group (1976) found that maternal anti-paternal (but not anti-third-party) MLR was depressed. Takeuchi (1980) could not find IgG blocking antibody in women with chronic spontaneous abortions, but detected an increase in antibody titer if the result of unsuccessful pregnancy was hydatidiform mole, a tumor of trophoblastic origin. Women with hydatidiform mole and other trophoblastic neoplasias have an increased frequency of HLA-sharing with their husbands

(Pattillo, 1980). Antisera (Faluk, *et al.*, 1978; McIntyre and Faulk, 1982; and McIntyre, *et al.*, 1983) and monoclonal antibody (McLaug[illegible] *et al.*, 1982 and Johnson, *et al.*, 1981) to TLX; the HLA-related trophoblast antigen also reacts with lymphocytes. This fact led some investigators to hypothesize that a mild humoral anti-class I response could stimulate placental and fetal growth, by generating antibody that would prevent a subsequent destructive cell mediated response (Faulk and McIntyre, 1981). Alternately, HLA antigens could be modulated off the trophoblast surface by specific antibody (Underwood, *et al.*, 1985, and Mowbray and Underwood, 1985). Thus, chronic aborters immunized with paternal (Beer, *et al.*, 1981 and Mowbray, *et al.*, 1983; 1985) or pooled thrid-party (Taylor and Faulk,1981) lymphocytes were able to complete gestation. In all cases the success of pregnancy was associated with the generation of anti-paternal antibody that inhibited an MLR. Procedural differences among the investigators, however, could have important implications. As Peter Johnson (1982) has suggested, the HLA sharing noted in the aborters is not dramatic and [illegible] related to different methods of HLA typing rather than true homozygosity. In fact, some groups (Lauritsen, Kistensen, and Grunnet, 1976; Harris and Lordon, 1976; Rocklin, *et al.*, 1982; Caudle, *et al.*, 1983 and Johnson, *et al.*, 1984) found no such HLA-compatibility among chronic aborters. Thus, Mowbray's group did not use HLA relatedness as a selection criterion in their studies, and found no correlations to class I identity in the results, but a significant HLA-DR sharing between abortion-prone couples (Mowbray and Underwood, 1985 and Mowbray, *et al.*, 1983). The other laboratories, however, included HLA sharing in the selection of women for treatment, in Beer's case with potentially dire consequences. Some of the offspring of the chronic aborters who conceived under immunotherapy developed intrauterine growth retardation, SCID, or chromosomal abnormalities (Beer, *et al.*, 1987). This is strong suggestive evidence that the failure of many conceptuses that are HLA matched to their mother is due to the action of homozygous recessive lethals (Gill, Siew and Kunz, 1983 and Gill, 1983) and that these malformations escape elimination under the protection of blocking antibodies. In support of this conclusion, it has been noted that the association of HLA-DR with spontaneous abortion indicates linkage disequilibrium with the target antigens since the class II antigens are not present in those areas of the placenta accessible to maternal effectors (Faulk and McIntyre, 1981 and Johnson, P. M., 1982). Also, Awdeh

and colleagues (1983) have found evidence for a human equivalent of the T complex which is centromeric to H-2 on murine chromosome 17, and the rat equivalent, the *grc* locus (Gill, Siew, and Kunz, 1983). A decreased MLR between the lymphocytes of couples experiencing chronic abortions has been noted by some investigators (Unander and Olding, 1983 and Lauritsen, Kristensen, and Grunnet, 1976) which may be linked to HLA-B homozygosity (Johnson, *et al.*, 1984).

Thus, it appears that absence of a protective immune response (blocking antibody?) whose generation is regulated by an unknown gene within the MHC leads to a failure of pregnancy, perhaps through the subsequent generation of cell-mediated immunity. However, blocking antibody is only detected in 50% of women with normal pregnancy, although antibodies with other specificities can be found (Anon, 1983). Hence, definitive conclusions cannot be drawn about the relationship between circulating anti-paternal antibody and successful pregnancy, or correlations between these antibodies and other effector mechanisms. Such studies are difficult in humans where the genetics and immunological status of the mating population cannot be controlled.

Blocking antibody may also play a role in successful equine pregnancy (Allen, Kydd, and Antczak, 1987). Early in gestation in the horse, the trophoblast invades the uterine stroma in discrete patches, the endometrial cups. The maternal reaction to this intrusion includes an accumulation of lymphocytes which isolates the fetal tissue from the surrounding maternal decidua (Allen, 1979). Anti-paternal MHC antibody appears shortly thereafter in the maternal serum (Antczak, Miller, and Remick, 1984). Ultimate degeneration of the cups later in pregnancy is preceeded by infiltration of the surrounding maternal lymphocytes. The antibody is not usually found in MHC compatible horse matings and is also absent in mares carrying mule conceptuses, pregnancies which usually fail. Embryo transfer of donkey conceptuses to horse foster mothers is accompanied by failure of endometrial cup formation; the leukocytic infiltrate occurs earlier and is of greater magnitude and distribution. Only ten percent of these pregnancies complete gestation, but they can be rescued either by passive transfer of serum (but not equine chorionic gonadotropin) from mares carrying normal conceptuses, or by paternal lymphocyte immunization prior to embryo transfer (Allen, Kydd and Antzack, 1987).

Pursuant to observations that CBA/J x DBA/2 matings had a high spontaneous abortion rate (Clark, McDermott, and Szewczuk, 1980), Chaouat, Kiger and Wegmann (1983) developed a murine model to study the effects of immunological intervention in spontaneous abortion. Immunization of CBA/J females with BALB/cCr (but not DBA/2J or CBA/J) spleen cells one week prior to mating with DBA/2J males could lower the resorption rate from 25% to 5%. The immunization generated cells suppressive for an anti-paternal MLR in the pregnant CBA/J female spleen and placenta that are not present in untreated, chronically aborting controls (Chaouat, Kolb and Wegmann, 1983). The decreased abortion rate is associated with the generation of an IgG_1 anti-paternal MHC antibody that can passively transfer protection to naive, virgin CBA/J females (Chaouat, *et al.*, 1985). Kiger and colleagues (1985) have further defined the relevant immunogen to an epitope(s) on the BALB/cCr (but not BALB.B, BALB.K, or DBA/2) male splenocytes (female cells do not immunize). Studies conducted by Tartakovsky (1985, 1987) suggest that this antigen may be characteristic of gestational tissue carrying the DBA/2J haplotype since intravenous administration of (C57Bl/6J x DBA/2J)/F_1 placental and fetal (but not spleen) cells will increase the spontaneous abortion in C57Bl/6J females mated to DBA/2J males.

Liver regeneration studies (Pliskin and Prehn, 1975) indicate that a cell mediated response may promote growth via lymphokines secreted by activated T-cells. A similar mechanism apparently acts to enhance tumor growth (Prehn and Lappe, 1971 and Prehn, 1983) and may also enhance placental growth during pregnancy, possibly explaining the increase in placental size observed by earlier workers. Spleen and peritoneal exudate adherent non-T-cells from syngeneically pregnant mice can stimulate proliferation of teratocarcinoma and cultured mouse blastocyst cells (Fenderson, Bartlett, and Edidin, 1983). Gudson (1971) observed that rabbit anti-rat thymocyte (but curiously, not anti-lymphocyte) serum severely reduces litter size in pregnant rats. Indeed, colony stimulating factor-granulocyte/macrophage (CSF-GM) purified from supernatants of EL-4, a murine T-lymphoblast, increases the proliferation and phagocytic capacity of esterase positive placental cells *in vitro* (Chauoat, *et al.*, 1987 and Athanassakis, *et al.*, 1987). Anti-thymocyte treatment of the pregnant females abrogated placental cell proliferation, but did not increase the resorption rate (Athanassakis, *et al.*, 1987). Supernatants of both human and

murine placental cells cultured in serum-free medium contained interleukin-1 and CSF-GM, but not interleukin-2, interferon, erythropoeitin or B-cell growth factor (Flynn, Finke, and Hilfiker, 1982 and Flynn, Finke, and Loftus, 1985). Placental cells have a high cell surface concentration of receptors for growth-promoting proteins, such as transferrin, epidermal growth factor, platelet-derived growth factor and insulin (Galbraith, *et al.*, 1980; Avruch, *et al.*, 1982; Richards, *et al.*, 1983; Carson, *et al.*, 1983; and Goustin, *et al.*, 1985). Nonetheless, it must be noted that nude mice (which have severely compromised T-cell responses) can be mated successfully in a gnotobiotic environment. It is therefore possible to speculate that the primary effect of immunostimulation on fetal viability occurs under conditions of microbial compromise of the pregnancy, and the effect is due to an increase in placental phagocytic capacity.

G. Perspectives

The above discussion has emphasized that a paradox exists during mammalian gestation. The maternal immune system can, and often does respond to paternal antigen present at the maternal-fetal interface, yet the fetus suffers no ill effects due to the generation of maternal anti-paternal immunity. In fact, the lymphokines produced by specifically activated T-cells may be a major source of placental growth stimulation. Fundamental questions about these interactions remain to be explained. In this thesis, I have investigated some of the afferent mechanisms involved in these interactions.

The nature of the antigenic stimulus for the maternal response remains unclear. MHC antigens are the chief mediators of sensitization for graft rejection and so would be the most likely immunogens. It is well established that placental and fetal membranes lack class II antigens. (However, antigen presentation is not defective due to a lack of Ia, since Ia positive cells are found in the decidua and fetal areas adjacent to the maternal-fetal interface.) The ontogeny and distribution of class I antigens has not been well defined. Class I expression in the whole conceptus is first detected on day 9 of gestation, and the early placenta (day 10) binds low levels of anti-H-2 antibodies. Autoradiography of placental sections shows that anti-H-2 antibodies localize to parietal endodermal cells, macrophage-like cells within the yolk sac, and to a

lesser extent, some cells scattered throughout the spongiotrophoblast. The placenta is a complex organ composed of many cell types, and it has not yet been possible to determine the lineage of these H-2 positive cells.

The possibility that non-traditional class I antigens are present in gestational tissue is implied from the studies of the Pa antigen in the rat by Gill and colleagues, and the observations that monoclonal antibodies to the conserved region of the class I molecule bind to some minor populations of human trophoblast while the traditional class I polymorphic determinants found on almost all nucleated cells are absent. Proteins encoded by most of the murine *Qa/Tla* genes have yet to be found. It is intriguing to speculate that the *Qa/Tla* genes might provide class I-like determinants of gestational tissue and an immunogenic stimulus to the maternal immune system. If the fetus itself lacks these determinants, then the conceptus could utilize the growth promoting properties of lymphokines while exposing the fetus to minimum risk. Therefore, a major part of this thesis was devoted to the study of the nature of the class I antigens at the maternal-fetal interface.

Protection of the fetus could also be facilitated by the physical barrrier of the placenta. Since the maternal and fetal circulations do not mix, cells and/or soluble substances can only traffic from the mother to fetus by passing through the placenta or fetal membranes. IgG can enter the fetus from the yolk sac after binding of the Fc portion to the yolk sac endoderm and internalization. Antibodies specifically bound via the Fab portion are blocked from passage and ultimately degraded. The trafficking of cells into the fetus is not as well understood. Normal pregnancy does not result in the appearance of maternal cells in the fetal tissues or circulation. Indeed, the development of SCID in young children is highly correlated with the presence of maternal cells and an ongoing graft versus host reaction. In mice, maternal white blood cells are rarely found in the fetus, while red cells frequently traffic across the placenta. However, many studies have used ambiguous markers or rather insensitive detection techniques. The development of noninvasive, nonmanipulative (glucose phosphate isomerase isozyme assay) and very sensitive (detection of fluoresceinated cells with the Fluorescence Activated Cell Sorter) techniques has enabled the re-evaluation of this important question in this thesis.

Materials and Methods

A. Mice

Inbred BALB/cCr (BALB, *Gpi-1*$^{a/a}$, *H-2* $^{d/d}$, *Qa-2*$^{a/a}$, *Tla*$^{c/c}$), C3H/HeJ (C3H, *Gpi-1*$^{b/b}$, *H-2*$^{k/k}$, *Qa-2*$^{b/b}$, *Tla*$^{b/b}$), (BALBc/CR x C3H/HeJ)F_1 (BC3/F_1, *Gpi-1*$^{a/b}$, *H-2*$^{d/k}$, *Qa-2*$^{a/b}$, *Tla*$^{b/c}$), C57Bl/10 (B10, *Gpi-1*$^{b/b}$, *H-2*$^{b/b}$, *Qa-2*$^{a/a}$, *Tla*$^{b/b}$), C57Bl/6 (B6, *Gpi-1*$^{b/b}$, *H-2*$^{b/b}$, *Qa-2*$^{a/a}$, *Tla*$^{b/b}$), A/J (*Gpi-1*$^{a/a}$, *H-2*$^{a/a}$, *Qa-2*$^{a/a}$, *Tla*$^{a/a}$), and (C57Bl/6J x A/J)F_1 (B6A/F_1, *Gpi-1*$^{a/b}$, *H-2*$^{a/b}$, *Qa-2*$^{a/a}$, *Tla*$^{a/b}$) mice used for transplacental trafficking and antigen expression by preimplantation embryo studies were obtained from the Laboratory Animal Breeding Unit at the University of Alberta (Ellerslie, Alberta). Inbred BALB/cJ (BALB), C3H/HeJ (C3H), and C57Bl/10J (B10) mice used for placental immunoabsorbant and Class I mRNA expression studies were obtained from the Jackson Laboratory (Bar Harbor, ME).

Matings were conducted by caging each virgin female overnight with a male and examining the following morning for the presence of a vaginal plug. Pregnancies were timed using the day of plug observation as day 0. Mice at the University of Alberta colony deliver on day 19.

B. Tissues

In order to examine transplacental passage of maternal cells into the fetus, embryos from days 15-18 of gestation were delivered by excision of the uterus and quick dissection of each embryo from its placenta and fetal membranes. Neonatal mice (day 19 = day of delivery, or +1 = one day postpartum) were born naturally and allowed to suckle. The animals were killed by decapitation and the liver (days 15, 16, or 17), blood and liver (day 18), or blood, spleen, and liver (days 19 or +1) taken. In all cases, blood was collected into phosphate buffered saline (PBS, pH 7.2) containing 1% heparin. Solid tissues were pushed through a fine stainless steel mesh screen into Leibowitz medium (L15, pH 7.2, Grand Island Biological Supply Company (GIBCO), Grand Island, N.Y.) to yield a single cell suspension. The preparations were washed once. Liver cell suspensions were further processed by layering each sample onto 14 % Ficoll-Isopaque and

centrifuging for 25 minutes at 600g, after which the interface population (highly enriched for mononuclear cells) was collected and washed. Cell suspensions were counted and diluted in PBS to a concentration that was constant within tissue types. The final number of cells obtained varied with kind and age of the tissue. In order to avoid obscuring isozyme bands in the starch gels, we used no more than 10^8 cells per sample. Thus, either an aliquot of neonatal blood, or the entire contents of other tissues, were processed for electrophoresis. The numbers of cells obtained from the various tissues were as follows: liver, 5 x 10^5-10^7; spleen, 10^6-10^7; blood, 10^7-10^8. One ml of each sample was then pelleted, resuspended in 50 µl of PBS, submerged in liquid nitrogen to lyse the cells, and stored at -70° C until electrophoresed.

Immature (dictyate) oocytes were obtained by teasing apart the ovaries of young (4-6 week) virgin females and collecting the large eggs with prominent germinal vesicles and minimal cumulus cell contamination. Preimplantation blastocysts were flushed from the uteri of females 3.5 days post-copulation (Rafferty, 1970). Oocytes and blastocysts were washed twice in L15 and transferred to a solution of pronase (300 units/ml in PBS, Calbiochem, La Jolla, CA). Zona pellucida dissolved in 3-10 minutes after which the oocytes or blastocysts were removed and washed three times in L15, then transferred to RPMI 1640 (GIBCO) and allowed to recover one hour at 37° C, 5% CO_2, in a humid incubator. Blastocyst outgrowth cultures were obtained by placing blastocysts flushed from the uterus directly in RPMI 1640 plus 10% fetal bovine serum (FBS). After 2-4 days in culture, the blastocysts had hatched out of the zona pellucida and "implanted" onto the surface of microchamber glass slides (Lux, Newberry Park, CA). These cultures displayed a typical morphology of tightly clustered round ICM cells growing atop the lawn of flat, fibroblast-like trophectodermal cells.

Two methods for obtaining single cell suspensions from gestational tissues were compared. Individual implantation sites of various gestational ages were teased away from the uterine wall and the decidual cap peeled off using fine forceps. Placentas were carefully clipped from yolk sacs, and the peri-placental area of yolk sac insertion discarded. The fetuses were freed from the remaining umbilicus and amnion by gently teasing and trimming. All tissues were kept in L15 on ice until processed. Fetal liver and/or spleen and thymus were removed as needed after decapitation of the fetus. Half of the

tissue thus obtained was pushed through a fine mesh stainless steel screen and, after a slow speed "quick spin" (15-30 second centrifugation) to remove the pelleted debris, washed three times in L15. The remaining tissue was minced, placed into 1 mg/ml collagenase (type IV, Sigma) plus 150 µg/ml DNAse (Sigma) in PBS, and incubated at 37° C in a shaking water bath for 45-60 minutes. This mixture was then vigorously resuspended, filtered through nitex gauze (Tetko, Inc., Elmsford, NY), "quick spun", and the resulting cells washed once in L15. Lysis of red blood cells was accomplished by hypotonic shock, followed by a further 2-3 washes in L15. The cell suspensions were evaluated on the basis of total yield and composition using trypan blue exclusion to reveal dead cells. Control adult spleen was similarly treated.

Gestational tissues used to examine *in vivo* localization of radiolabeled anti-class I monoclonal antibodies were similarily dissected. However, in these experiments, the maternal circulation was first flushed through the left ventricle with 30-50 ml heparinized saline to reduce background radioactivity.

C. Electrophoretic analyses

GPI is a ubiquitous enzyme that exists in two allelic forms which can be resolved by starch gel electrophoresis. Mice that are homozygous for the *Gpi-1*^{b} allele express an isozyme that migrates more cathodally (faster) in an electric field than the form expressed by the *Gpi-1*^{a} mice; heterozygotes (*Gpi-1*$^{a/b}$) express both these bands, as well as a heterodimeric band with intermediate mobility.

GPI isozymes in the cell lysates were separated by starch gel electrophoresis and developed by the nitrocellulose overlay technique (Peterson, et al., 1978 and Tsuyuki, et al., 1966) as modified by Hunziker, Gambel, and Wegmann (1984). Briefly, between 0.3 and 0.6 µl of sample was dispensed from a fine capillary pipet into a well cut into a gel slab made from 17% electrostarch (lot 3307, Electrostarch Co., Madison, WI). The running conditions were 100 volts for 10 minutes followed by 220 volts for 5 hours, at 4° C. Each gel was then cut to fit into a 5 x 60 mm petri dish and overlaid with a nitrocellulose filter (47 mm diameter, 0.20 µm pore size, Millipore Corp., Bedford, MA). After the filter was uniformly wet, an O-ring, covered on the "down" side with high vacuum grease, was applied to form a well. The

components of the staining solution were: 10 µl glucose-6-phosphate dehydrogenase (1 mg/ml, Boehringer Mannheim, Gmb, FRG); 5.3 ml stain buffer (20 mM citrate, 30 mM $MgCl_2$, pH 8.0 with Tris base); 1.0 mg NADP, sodium salt (Boehringer Mannheim); 2.0 mg D-fructose-6-phosphate (Boehringer Mannheim); 0.4 mg phenazine methosulfate (PMS, Sigma); 1.0 mg nitroblue tetrazolium (NBT, Sigma). These were added in the order listed to a light-shielded bottle, allowed to dissolve, and immediatedly poured into the well. Discrete bands began to appear by 20-30 minutes. The gels were developed for 4-7 hours (depending upon the type of sample) to allow visualization of F_1 bands at the 1% level.

Quantitative measurement of the amount of trafficking was determined by scanning the stained nitrocellulose filters (mounted on microscope slides) in the reflectance mode of a Electrophoresis Scanner, Model 80100 (Camag, Muttenz, Switz.). The signal produced was analysed in a Hewlett-Packard Model 3390A Reporting Integrater (Palo Alto, CA), which determined the area of each band as a percentage of the total pattern. Using this method, the sensitivity of the system was the resolution of 1% of the minority isozyme (the F_1 pattern) in an artificial heterozygous:homozygous mixture of 10^5 cells. Thus, values obtained were always compared to a set of concurrently electrophoresed standards of artificial mixtures that were made from the same tissue types and cell concentrations as the experimental samples assayed.

D. FACS analyses

RBC obtained from the periphery, or spleen cells from BALB females were collected as described above. Leukocytes were not removed from the RBC suspension. Splenic white cells (WSC) were prepared by resuspending pelleted spleen cells in 0.83% NH_4Cl-Tris base, pH 7.2, allowing the suspension to stand at room temperature for 8 minutes and finally washing three times with Leibowitz media.

RBC and WSC were labeled by incubation with fluorescein isothiocynate (FITC, Isomer I, Sigma) in binding medium, as per Butcher and Weissman (1980) for 30-45 minutes at 37° C. Labeled cells were separated from free fluorochrome by centrifugation through fetal bovine serum. The pellet was washed three times and resuspended in PBS to a final concentration of 10^8 cells/ml for WSC and 10^9 cells/ml for RBC. Using this method we produced a

stock solution of cells that was >99% labeled although about 10% of the cells were only lightly labeled.

One ml of cell suspension was administered intravenously to syngeneically mated BALB females on day 18. Twenty-four hours later a sample of maternal blood and spleen, and either the neonatal liver (when WSC had been injected) or neonatal blood (when RBC had been injected) were collected from each animal. After washing in PBS, single cell suspensions were made in 10% formalin-PBS, and the fixed samples analysed on an Ortho 50H FACS. Fluorescent cells appeared in two separate peaks corresponding to a weakly labeled (channels 25-150 for blood and spleen samples, channels 35-300 for liver samples) and intensely labeled (channels 150-1000 for blood and spleen samples, channels 300-1000 for liver samples) when read at a photomultiplier tube maximum of six volts. This high voltage increased the efficiency of detection to about 1 in 10^5 cells. Magnitude of the labeled RBC transfer was calculated using the formula:

$$\%\ \text{labeled RBC in fetus} = \frac{\%\ \text{labeled cells in fetal blood} - \%\ \text{labeled cells in fetal blood of uninjected control}}{\%\ \text{labeled cells remaining in maternal blood}} \times 100$$

in which the negative control, the value for the "percent labeled cells in fetal blood of uninjected control", provided a measure of machine background across the different cell types. The amount of WSC trafficking was estimated using a similar formula. The percent of labeled cells remaining in the maternal white blood cell pool was estimated using our reported value for the percent of labeled cells remaining in whole blood (see Results).

E: Antibodies

Antisera used in these investigations were NIH typing reagents raised in congenic combinations and absorbed to limited specificity. The E-28b antiserum recognizes H-2D^b, E32 is specific for H-2D^k, and D13(2) is specific for H-2D^d. All antisera were titered using an indirect immunofluorescent assay on BALB, B10, and C3H splenocytes.

Hybridomas producing anti-class I monoclonal antibodies (Table 1) were obtained from the American Type Tissue Collection (Bethesda, MD) as

confluent cultures or frozen stocks. The potency and specificity of culture supernatants was confirmed with a fixed cell radioimmunoassay (RIA, Mishell and Siigi, 1980). Briefly, 10^6 splenic white blood cells (red blood cells removed by hypotonic shock) were diluted in PBS and added to each well of a flat bottom microtiter 96-well plate pre-coated with poly L-lysine (PLL, 1-2 mg/100 ml in PBS, Sigma). The plates were centrifuged at 300 g for 5 minutes to pellet the cells to the bottom of the wells and then fixed at 4°C in 0.25% gluteraldehyde in PBS. After four washes in PBS, the plates were flooded with PBS plus 1% bovine serum albumin (BSA, Sigma) and 0.02% sodium azide, sealed in saran wrap, and stored at 4° C until use. Before each assay plates were examined microscopically for the presence of a confluent lawn of round cells. The storage buffer was shaken out and replaced with 5% normal goat serum (NGS) in PBS plus 0.05% Tween 20 (Sigma). After 30 minutes at room temperature to block Fc receptors, the plate was washed in RIA buffer (1% NGS, 0.05% Tween 20 in PBS). All subsequent dilutions, incubations, and washes were performed in RIA buffer. Test monoclonal antibodies were centrifuged at high speed (16,000 g) in an Eppendorf microfuge to pellet protein aggregates and diluted in RIA buffer, then added to wells. Positive and negative control antibodies as well as wells receiving RIA buffer alone were included in every assay. After 60-90 minutes at 37° C, the plates were washed three times. 50,000-100,000 cpm of ^{125}I-goat anti-mouse IgG (goat anti mouse IgG was obtained from BIOCAN Scientific, Inc. Mississauga, ONT and labeled via the chloramine T method of Greenwood, Hunt, and Glover, 1963) was added to each well. The plates were incubated an additional 60-90 minutes at 37° C, washed four times and the individual wells counted in a Rackgamma counter (LKB Instruments, Rockville, MD). Titer was recorded as the last dilution producing significant binding of the iodinated second antibody above control levels.

Hybridoma ascites was produced in pristane-primed mice, filtered through gauze and 0.45 µm millipore filters (Millepore Corporation) and tested for activity in fixed cell RIA. The placental immunoabsorbent assays required antibodies of high specific activity. Therefore, antibodies were purified from ascites fluid by affinity chromatography. A 15 ml Protein A-Sepharose (Pharmacia, Sweden) gel column was prepared and washed thoroughly with 50 ml of PBS. Ten ml of ascites fluid was passed through the column. The column was then washed with 50 ml of PBS, after which the effluent contained no more detectable protein. Twenty ml of 1M acetic acid was passed through

the column to elute the adsorbed immunoglobulins. The eluted solution was adjusted to pH 7 with Tris base. This purified solution of immunoglobulins was dialyzed against 3 changes of PBS at 4°C and the protein concentration determined by measuring absorbance at 280 nm (OD_{280} = 1.4 for 1 mg/ml IgG).

F. Radiolabeling of antibodies

^{125}I-labeling of antibodies was performed by the chloramine T method of Greenwood, Hunt, and Glover (1963), at a molar ratio of one iodine atom to one IgG molecule. 5 millicuries of radiolabeled sodium iodide (NEN Canada, Lachine, Que.) were added to a 200 microgram (µg) solution of the immunoglobulins in PBS. Fifty µl of chloramine T (2 mg/ml) were added to the mixture and the oxidation reaction allowed to continue for 2-5 minutes on ice. Further oxidation was stopped by neutralizing the mixture with 50 µl of sodium metabisulfite (6 mg/ml). Ten µl of 1% potassium iodide were added and the solution dialyzed against PBS to remove free iodine.

Monoclonal antibody was intrinsically labeled with ^{35}S-methionine. 20-8-4S or 28-8-6S hybridoma cells were grown *in vitro* in RPMI 1640 to a density of 10^6 cells/ml and washed three times in methionine-free RPMI. The cells were then suspended in 3 ml of methionine-free RPMI (10^6 cells/ml) and thus starved for 4 hours at 37°C, 5% CO_2. One millicurie of ^{35}S-methionine (NEN Canada) was added to each suspension and the cells incubated for six hours at 37°C with intermittent shaking. The cells were then spun at 500 g for 5 minutes and the supernatant containing the ^{35}S-anti H-2^b dialyzed against PBS to remove free ^{35}S-methionine.

G. Purification and assay of monoclonal anti-H-2 antibodies

^{125}I-labeled anti-H-2 antibodies were purified by absorption/elution by glutaraldehyde-fixed B10 spleen cells. 10^9 splenic white blood cells at a concentration of 2 x 10^7 cells/ml in L15 were mixed with an equal volume of 0.25% glutaraldehyde with continuous stirring over a period of 5 minutes. The cells were then washed in L15 containing 0.1M glycine (to bind excess glutaraldehyde), resuspended in L15 plus glycine, and left at room temperature for 30 minutes. After two washes in PBS, an equal volume of radiolabeled antibody was added to the cell pellet and the mixture incubated for one hour at 4° C. The cells were washed twice with PBS and once with saline. The antibodies that were bound to the cells were eluted by adjusting the pH to 3.2

with 0.1 M acetic acid, and the cells centrifuged immediately. The neutral pH of the supernatant was restored by adding solid Tris base. The supernatant, consisting of specific ^{125}I-labeled antibody was then tested in a quantitative absorption assay.

Radiolabeled antibodies were assessed for specific activity by comparing their binding to target versus control spleen cells. Single splenic white blood cell suspensions were prepared and then suspended in L15 plus 1% normal mouse serum (NMS) were prepared in doubling dilutions ranging from 50 x 10^6 to 1.5 x 10^6. 5,000 to 50,000 cpm of labeled antibody were mixed with the cells and incubated for one hour at 4°C. The cells were then washed three times, and the radioactivity in the resultant pellets measured in the Rackgamma counter. The specific activity (binding) was defined as cpm on target cells (at the plateau of the binding curve) minus cpm on control cells, expressed as a percent of total cpm added to the cell suspension.

H. Indirect Immunofluorescence

The binding of antisera and monoclonal antibodies to oocytes, blastocysts and implants was assessed by indirect immunofluorescence (Heyner and Hunziker, 1979). The medium used throughout was RPMI 1640. Oocytes or blastocysts were added to appropriate dilutions of test antibody (1:10 for sera and 1:500 to 1:1000 for ascites). The culture supernatant of blastocyst outgrowths was replaced with RPMI without FBS for 20 minutes, and this was replaced with the diluted antibody solution. Antisera or ascites of irrelevant specificity provided negative controls. After 30 minutes incubation at 37° C, the cells or tissues were washed three times in RPMI and then incubated with a 1:10 dilution of FITC-goat anti-mouse IgG (Tago, San Francisco, CA) for an additional 30 minutes at 37°C. After three washes the oocytes, blastocysts, or implants were examined microscopically using a Zeiss microscope fitted with an epi-fluorescence system.

I. Antibody Binding to Gestational Tissues

Estimation of the number of class I antigens on gestational tissue accessible to anti-class I antibody circulating in the maternal circulation was determined by measuring the level of iodinated antibody found in embryonic and extraembryonic tissues following intravenous injection of the mother. C3H

females were mated to B6 (target) or C3H (control) males and the mothers injected with approximately 10^6 cpm purified, iodinated, absorbed/eluted monoclonal antibody on day 15. Six hours later the mothers were anesthetized and their circulations flushed with an excess of heparin-PBS as previously described. Decidual caps, yolk sacs, placentas, and embryos from each litter were pooled into heparin-PBS, weighed, and washed twice in heparin-PBS. The pooled tissues were counted in the Rackgamma counter and the results tabulated as cpm per gram of tissue.

J. Preparation of total cytoplasmic RNA

1. Dot Blots. Preliminary estimation of class I messenger RNA content of gestational tissues was accomplished using crude cytoplasmic suspensions dot blotted onto nitrocellulose (White and Bancroft, 1982). Tissue was obtained from dissection of target (B10 X BALB) or control (B10 X B10) implantation sites and processed fresh or from samples just thawed from -70°C storage. 0.1-0.2 g of tissue (wet weight) was added per ml of dot blot lysis buffer (10 mM Tris, 1 mM EDTA, 0.5% NP-40 (Sigma), pH 7.2) and the mixture homogenized for 60-90 seconds to rupture cell membranes (nuclear membranes are relatively insensitive to these conditions). After a 5 minute high speed (16000 g) centrifugation to pellet membranes, nuclei and other debris, the supernatant (cytosol) was mixed with an equal volume of a fresh solution of three parts 20X SSC (1X SSC = 0.15 M NaCl, 0.015M sodium citrate, pH 7.2) two parts 37% formaldehyde, and incubated for 15 minutes at 60° C. The solutions were then diluted in 15X SSC, first to 1:10 and in tripling dilutions thereafter. The diluted solutions were suction-adsorbed onto nitrocellulose sheets (first wet in H_2O, then in 15X SSC) and mounted into a 96-well minifold apparatus (Schleicher and Schwell, Keene, NH). Water and salts freely pass through the nitrocellulose but protein and nucleic acids are retained on the filter. The sheets were then baked for 24 hours at 90° C to afix the RNA to the nitrocellulose, and then hybridized to the appropriate ^{32}P-labeled oligonucleotide probe.

2. Northern Blots. Purification and size separation of total cytoplasmic RNA was necessary to detect the presence of specific class I messages. Tissues were obtained and prepared as for dot blots except that the lysis buffer was 0.14 M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris (pH 8.5), 0.5% (w/v) NP-40 plus 5 mM vanadyl ribonucleosidase inhibitor complexes. After high speed

centrifugation (16,000 g) for 5 minutes to pellet debris, an equal volume of 2X proteinase K buffer (0.2 M Tris (pH 7.5), 25 mM EDTA, 0.3 M NaCl, 2% (w/v) SDS) plus 200 µg/ml proteinase K were added, and the solutions incubated for 30 minutes at 37° C. Any remaining cytoplasmic protein and enzyme were removed by extracting the solution with an equal volume of a 50:50 mix of neutralized phenol and chloroform (Maniatis, Fritsch, and Sambrook, 1982). Separation of the organic phase (containing the proteins) and the aqueous phase (containing the RNA) was accomplished by high speed centrifugation for 15 minutes. The extraction was repeated to assure completion.

The RNA was further purified and concentrated by precipitation. 0.1 volume of 3 M sodium acetate plus 2.5 volumes of 98% ethanol were added to each sample which was then stored overnight at -20°C to insure complete precipitation. The insoluble RNA was pelleted at 16,000 g for 15 minutes, washed in 70% ethanol and centrifuged again. The resultant pellet was air dried (or lyophylized) and resuspended in 50-500 µl of TE (10 mM Tris, 1 mM EDTA, pH 7.4). RNA concentration was estimated by absorbance at 260 nm (40 µg/ml RNA ≙ 1.0 OD_{260}). Samples were stored in TE at -70°C, or in 80% ethanol at -20° C if long term storage was necessary. RNA was fractionated by agarose gel electrophoresis (Maniatis, Fritsch, and Sambrook, 1982). Briefly, 20 µg of RNA was diluted in 16 µl of buffer (0.01 M NaH_2PO_4 (pH 7.0) plus 50% DMSO and 0.2 M glyoxal) and denatured at 50° C for 30 minutes. The samples were mixed with 4 µl of loading buffer (0.01 M NaH_2PO_4 (pH 7.0) plus 50% glycerol and 0.4% bromophenol blue), loaded into the wells of a 1.4% agarose gel and electrophoresed (negative to positive) at 5 volts/cm of gel for 60-90 minutes in TBE (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA). The gels were stained for 60 minutes in 1 µg/ml ethidium bromide in water and examined under short wavelength ultraviolet light. Examination of the location and intensity of the ubiquitous 18S and 28S ribosomal RNA bands confirmed the concentration and established the quality of the sample.

The RNA was transferred to nitrocellulose by the Northern blot procedure as described by Maniatis, Fritsch, and Sambrook (1982). After 24 hours the nitrocellulose filters were baked at 90°C for 24 hours before hybridization to oligonucleotide probes.

K. Oligonucleotide probes.

The class I specific oligonucleotide probes were derived in the laboratory of Leroy Hood, California Institute of Tecnology, Pasedena, CA, from a BALB/c overlapping genomic cosmid library (Steinmetz, *et al.*, 1981) that has been shown by restriction enzyme mapping to contain all the genes present in the BALB MHC (Winoto, *et al.*, 1983 and Fisher, *et al.*, 1985). After subcloning the 3' end of each gene into the M13 plasmid vector (Hunt, *et al.*, unpublished), and using an oligonucleotide primer derived from the 3' end of the fourth exon (conserved across class I genes), each gene was sequenced through the transmembrane region via the chain termination method (Sanger, *et al.*, 1980). The high degree of variability in the transmembrane excn was used as a basis for the construction of probes unique to each gene. Oligonucleotides 16-21 residues long were chemically synthesized (Beaucage and Caruthers, 1981), purified, and concentrated to 10-100 ng/µl. Each probe was checked for specificity by hybridization to restriction enzyme digests of the cosmid library, and found to bind to one to five genes at high stringency (Table 2). The above collection of well-characterized molecular probes was supplied to us by S. Hunt (California Institute of Technology).

Before hybridization to the nitrocellulose filters containing RNA, the probes were labeled to high specific activity using the exchange reaction of polynucleotide kinase (PNK) and ^{32}gamma-P-ATP. A 50 µl cocktail containing 20 units of PNK (BRL, Bethesda, MD), 1 µl of oligonucleotide probe and 100 µCi ^{32}gamma-P-ATP (Amersham Canada Ltd., Oakville, ONT) in 10 mM $MgCl_2$, 0.1 M Tris (pH 7.6), 20 mM beta-mercaptoethanol, was incubated at 37°C for about 60 minutes, or until at least 75% of the label was incorporated into the probe as assessed by paper chromotography using Whatman DE81 paper and 0.5 M formate. Labeled probe was separated from labeled free nucleotides by column chromatography through G-25 Sephadex and purity assessed on a 20% PAGE gel run at 500 v for one hour and then exposed overnight to Kodak XAR-5 film. Fractions containing only labeled probe were pooled and an aliquot counted in a Rackbeta counter.

L. Hybridizations

Nitrocellulose filters containing RNA from dot blot adsorption or Northern transfer were wet in 3X SSC and then prehybridized in 20 ml of hybridization buffer (5X SSC, 5X Denhardt's solution (Maniatis, Fritsch, and Sambrook, 1982), 0.1% SDS, 5 mM EDTA, 150 µg/ml heat denatured salmon sperm DNA) in sealed plastic bags immersed in a 37°C water bath for at least 6 hours. The filters were transferred to fresh plastic bags containing at least 2.5 ml/filter of hybridization buffer plus 25% dextran sulfate and 10^6 cpm/ml purified ^{32}P-labeled probe. The sealed bags were incubated 12-18 hours in a 37°C water bath. The filters were then rinsed in wash buffer (2X SSC, 0.1% SDS), washed twice in wash buffer at 37°C for 15 minutes, air dried, mounted and exposed to Kodak XAR-5 film in a light-tight cassette with an intensifying screen. After 24 hours the film was developed and the filters either re-exposed for 4 days or washed again at higher stringency (2 washes at 45°C) and then re-exposed. In some instances a higher stringency wash at 60°C followed by a 10 day exposure was also done.

Filters were reprobed 2-3 times after stripping off the previously used probe by three 20 minute washes in 0.1X SSC at 90°C.

Results

A. Trafficking Studies

A fundamental question underlying any investigation of maternal anti-fetal responses is whether or not the maternal effectors (cells, antibodies, or lymphokines) have access to their fetal targets. Previous work in Dr. Wegmann's laboratory has shown that anti-class I antibody is specifically absorbed by target placentas and thus denied access to the fetus, although others have shown that this absorption is limited (Bell and Billington, 1983c and Adeniyi-Jones and Ozato, 1987). The issue of transplacental traffic of maternal cells has not been resolved; and yet the potential for tissue destruction by an agressive cell mediated response is much greater than the possible damage caused by antibody. This is especially true in an immature animal lacking such accessory molecules as complement which mediate the destructive effects initiated by specifically bound antibody. The cascade of effects ellicited by cell mediated immunity makes the presence of just a few sensitized lymphocytes a dire potential threat to the immunoincompetent fetus. Not only does the focus of a cell-mediated reaction spread due to the accumulation of breakdown products from dead and dying cells in the immediate vicinity, but, more importantly, lymphokines elaborated by specific T-cells can recruit and activate even more lymphocytes to increase and spread the response. Thus, it is critical to be able to detect even a very low level of maternal to fetal cellular traffic.

Two methods of evaluating transplacental cellular traffic were used in the work presented in this thesis. Firstly, I examined the presence of maternal cells in fetal tissues using the allelic polymorphisms of the electrophoretic mobility of a ubiquitous enzyme marker. This enabled evaluation of trafficking in an intact system: the mothers underwent no experimental manipulation that might change the architecture of the fetal-maternal relationship. The technique was reliable and accurate but not as sensitive as necessary to detect very small numbers of transplacental migrants. In order to increase this sensitivity, we utilized the Fluorescence Activated Cell Sorter. Fetal tissues were scanned for the presence of fluoresceinated cells that had been previously injected into the maternal circulation. The limit of the FACS under the experimental conditions used was $1:10^5$ cells.

1. GPI analysis. When an F_1 female is backcrossed to a male of one of the paternal strains and a trait controlled by one codominant locus is examined, half of the offspring should display the maternal (F_1) type and the other half should have the paternal pattern. We used two such kinds of backcross, namely CC3/F_1 female x C3H male, and B6A/F1 female x J male. A total of 334 offspring from 34 litters were analyzed for allelic expression of GPI. As expected, half of the offspring were *Gpi-1*$^{a/b}$ and expressed the 1:2:1 ratio of slow:intermediate:fast bands that is indicative of a genetic F_1 hybrid. These samples were discarded as it would be impossible to distinguish any maternal F_1 cells in them.

The remaining samples were phenotypically homozygous for *Gpi-1*^{a}. Results of the electrophoretic analyses of these remaining samples are shown in Table 3. Each lysate was examined at least twice to verify the result, and run concurrently with standards of 0, 1, 2.5, and 5% *Gpi-1*$^{a/b}$ cells in a *Gpi-1*$^{a/a}$ cell suspension (Figure 7).

170 of 172 homozygotes tested showed no evidence of transplacental migration of maternal cells. However, two of these animals (one from day 18 in the CC3/F_1 matings, and one from day 19 in the B6A/F1 matings) displayed a high percentage of maternal cells (35% and 25%, respectively) in their livers. Both were assayed as part of a pilot study in which only the livers of one litter from each strain combination were examined. Therefore, we could not study the distribution of maternal cells in other organs. There was no apparent abnormality in these two mice or their littermates.

2. FACS analysis. In order to increase the sensitivity of our detection techniques, we scanned neonates for the presence of FITC-labeled blood cells that had been injected into the maternal circulation one day before parturition using the FACS. Butcher and colleagues (Butcher and Weissman, 1980 and Butcher, Scollay, and Weissman, 1980) have shown that direct fluorescein labeling of cells under the benign conditions used here can produce intense fluorescence without affecting viability, migration, homing, or other functions of the cells. Using the protocol of these investigators, we incubated RBC and WSC in FITC to produce a population of fluorescent cells that were 90% intensely and 10% weakly labeled. The intensity and distribution of the label persisted over the 24 hour period that the cells were circulating through the

mother, since a stock of labeled cells (kept at 4°) did not lose fluorescein as determined by FACS scanning. Butcher and Weissman (1980) have noted that although the flourescent intensity of the cells decays at 37°, the cells are readily detectable for days after labeling.

A major theoretical source of error in this kind of study was the possibility of release of the FITC from a labeled, injected cell and subsequent uptake by an unlabeled, resident cell. We attempted to estimate the magnitude of this effect by injecting cells that had been labeled and then lysed by freeze-thaw. The maximum secondary labeling possible *in vivo* would then be revealed by FACS sorting of these negative controls. There was probably a small (but detectable) *in vivo* redistribution of the RBC label since the freeze-thaw injection produced counts above background in maternal blood and spleen (Table 4, lines 5, 6, 14 and 15). Most of these counts, however, were due to faintly labeled WSC (Table 4, lines 5 and 6). The reincorporation was probably higher in the fetal liver since the percent labeled cells detected in the livers of neonates, whose mothers had been given lysed WSC, was about twice that of the labeled cells detected in the negative (uninjected) controls (Table 4, line 16).

Thirty neonates from three litters were examined for the presence of transplacental passage of labeled RBC previously introduced into the maternal circulation. The average number of intensely labeled cells present in the blood of these animals was 0.047% (Table 4, line 10). Using the formula for estimating RBC trafficking, we found that this percentage was equal to 5.6% of the proportion of labeled blood cells that remained in the maternal circulation after 24 hours ([(0.047 - 0.0012)/0.81] x 100). Three animals, however, contained much higher numbers of labeled cells (Table 4, lines 11, 12 and 13). If these animals are excluded form the calculations, the average number of intensely labeled cells reduces to 0.0044%, which is 0.40% of the proportion of labeled blood cells remaining in maternal circulation after twenty-four hours. The scatter of the individual values of labeled RBC detected was quite large (Figure 8), and one third of the animals contained levels of intensely labeled cells exceeding three standard deviations above the mean of the levels in uninjected controls.

Twenty-four neonates (randomly-selected from four litters) were screened for evidence of WSC trafficking. We detected no significant evidence of maternal to fetal passage of cells in most of these neonates, though the

numbers of labeled cells remaining in the mother's blood was high (Table 4, line 17). One animal contained numbers of labeled WSC clearly greater than three standard deviations above the mean, and another neonate contained numbers of labeled WBC just at that level of significance (Figure 8).

The average number of intensely labeled white cells present in the fetal livers was 0.0086% (Table 4, line 9). The percent cells transferred was not directly estimable since we did not have a figure for the percent of labeled WSC remaining in the maternal lymphocyte pool, but rather a measure of labeled WSC as a percent of total blood. Since leukocytes usually represent approximately 0.1 - 0.2% of the cellular portion of the blood (Bannerman, 1983), we assumed that the 0.51 % of total cells labeled in the mother after 24 hours represented at least 72% of total white cells available for transplacental transfer ([0.51/ (0.51 + 0.2)] x 100). (According to Bannerman (1983)., a BALB female contains about about 10^7 circulating WBC. As we injected 10^8 labeled WSC, the estimate of 72% of total cells labeled seems quite reasonable.) Therefore, we calculated that no more than 0.0044% of cells were transfered ([(0.0086 - 0.0054)/72] x 100).

The sensitivity of the FACS studies enabled us to establish the upper limit of naive maternal WBC trafficking into the fetal liver at 1 in 2 x 10^4 cells. The precursor frequency of cells from naive animals capable of proliferating in response to alloantigenic stimulation is about 1:500 to 1:2500 depending on the responder and stimulator strains used (Miller, Teh and Phillips, 1977; Ryser and MacDonald, 1979a, b). In our hands, the naive BALB mouse had a precursor frequency of 1:1200 C3H specific proliferating cells as assessed by a standard limiting dilution assay (performed by S. McCarthy). Therefore, it was theoretically possible that a few of the potential transplacental migrants were capable of initiating an allograft rejection. Sensitized maternal anti-fetal lymphocytes could be more aggressive and successful in their attempts at transplacental passage due to their activated state, or alternately, be trapped by encountering their relevant paternal antigens within the placenta. Addressing this issue was not technically feasible since priming the BALB mice increased the precursor frequency of 1:1200 to 1:400. Although this change produced significant effects in the *in vitro* assay, it provided insufficient enrichment to observe differences in the number of transplacental migrants.

Nonetheless, these results indicated the importance of examining the nature, location, and ontogeny of the paternal antigens at the maternal-fetal interface.

B. Immunofluorescence Studies of Pre- and Peri-Implantation Embryos

Having confirmed that immunologic effector cells were for the most part excluded from transplacental passage, we next decided to investigate the nature of the immunogenic (or at least antigenic) determinants found in the placenta and fetal membranes. As has been discussed, the mother is capable of, and often mounts, immune responses against paternal and trophoblast antigens, however it is not clear whether or not these responses are relevant to fetal survival. Indeed, the location, and, in some cases, even the very presence of transplantation antigens, those epitopes responsible for indicing a graft rejection response, has not been established. It is important to determine the ontogeny of the expression of the relevant antigens, since gestation has a precisely prescribed and limited duration. Effectors generated close to parturition may have insufficient time to affect the fetus. Sensitization during the fetal bleed at parturition would have no consequences on the fetus, and, if the determinants released into the maternal circulation are found only in fetal tissues (and not gestational membranes), also have no consequences on subsequent pregnancies in which fetal tissues are not exposed to maternal blood. Thus, we decided to investigate the ontogeny of class I antigen expression.

Zona-free oocytes (18-25 per experimental group), blastocysts (5-12 per group), or blastocyst implants (2-5 per group) were examined for the presence of detectable class I antigen expression using an indirect immunofluorescence assay (Table 5). Prior to use all antisera and/or monoclonal antibodies were specificity screened in an indirect immunofluorescence assay on a panel of B10, BALB and C3H spleen cells. Antisera bound to 50% of the target spleen cells up to a 1:270 (NIH anti-H-2^b or anti-H-2^d) or 1:30 (anti-H-2^k) dilution. Fluorescence of only 15-20% of the control (no first antibody) splenocytes was similar to that seen with normal mouse serum. Results of monoclonal antibody titering were more clear-cut as greater than 90% binding was achieved beyond titrations of 10^{-6} dilutions while controls were less than 20% fluorescent.

The antisera did not bind to oocytes. The anti-H-2^d antiserum bound weakly to trophoblast cells of blastocysts and did not bind to trophoblast cells of implant cultures. However, anti-H-2^d labeled the outer layer of cells delaminated from the ICM (presumably the primitive endoderm) of BALB or C3H blastocyst implants equally well. Anti-H-2^k reacted strongly with C3H blastocytsts, but was not assayed on the other strains. H-2^k was not detected in implant cultures, but these cultures also did not contain endoderm-like cells. Four different anti-class I monoclonal antibodies failed to react with oocytes or blastocysts even though the antibodies bound at high dilutions to spleen cells. Two different monoclonal antibodies specific for the B10 allele of beta$_2$ microglobulin were also tested. Neither of the antibodies reacted with oocytes. S19/8 was positive on blastocysts but 23-1.16 failed to react.

C. Immunoabsorbance of anti-class I antibodies.

The results obtained in the immunofluorescent assay suggested that the anti-class I antisera recognized some determinant on blastocysts that was not reactive with the monoclonal antibodies used in this study. The observation that the blastocysts were possibly reacting with anti-beta$_2$ microglobulin pointed to a class I-like antigen that was cross reactive with the antigens recognized by the antisera. Several groups of workers have shown by antibody (Sharrow, *et al.*, 1984) and CTL (Rigueora, *et al.*, 1983 and Cook, *et al.*, 1983) reactivity that H-2K/D antigens and determinants encoded by genes located in the Qa/Tla region of the MHC, are cross reactive. It has been shown that 20-8-4S (an antibody raised against C3H.SW splenocytes) also recognizes a Qa determinant found in B10.A mice, whereas 28-8-6S, a monoclonal antibody generated from the same immunization reacts only with the H-$2K^b$ and H-$2D^b$ molecules (Sharrow, *et al.*, 1984). Thus, it was decided to assess the specific binding of these antibodies to gestational tissue. The IIF studies proved difficult to quantify and so were not continued. However, the binding of other monoclonal antibodies to later stage placentas*in vivo* had already been measured (Wegmann, *et al.*, 1980) and so we used this technique to measure the level of class I antigens available to circulating antibody in gestational tissue. Both antibodies were of the same subclass and had the same light chain isotype so that their Fc receptor binding affinities should be close. Indeed, the non-specific absorption patterns seemed to be similar. Ascites from hybridoma 20-8-4S and 28-8-6S

were protein-A purified and concentrated to 1.6 mg/ml and 300 ug/ml respectively. 200 ug of antibody was ^{125}I-labeled and dialyzed against PBS. These preparations were tested by direct radioimmunoassay (RIA). The specific activity of 20-8 was 60% on both B10 and BALB with negligible binding to C3H splenocytes. However, 28-8 showed only 25% specific binding (C3H and BALB were both negative). These labeled preparations were then absorbed and eluted from glutaraldehyde-fixed B10 splenocytes. Specific activities were 61% for 20-8 and 30% for 28-8. The low levels of 28-8 binding lead to the belief that the labeling procedure altered the antibody's binding affinity, perhaps by derivitizing a tyrosine within the binding site. The antibodies were then labeled in a different way by culturing the hybridomas in medium containing ^{35}S-methionine. These intrinsically labeled antibodies were tested by direct binding to target and control cells. The specific activities did not dramatically change: specific activity of 20-8 was 67% and that of 28-8 was 15%. Since gamma radiation is more easily and accurately measured in whole tissue than beta rays, the ^{125}I-labeled antibodies were used for further assays. Fresh aliquots of antibody were labeled and evaluated. The specific activities of the absorbed/eluted preparations were: 75% for 20-8 and 27% or 46% for two different preparations of 28-8. 4 x 10^5 cpm of 20-8 (3 x 10^5 specific cpm) and 5 x 10^5 cpm (2.3 x 10^5 specific cpm for the 46% specific activity batch) or 7 x 10^5 cpm (2 x 10^5 specific cpm for the 27% specific activity batch) were injected into the tail vein of target (C3H x B10) or control (C3H x C3H) mice on day 15 of gestation. After 6 hours the mice were perfused, sacrificed, and the placentas, yolk sacs, embryos, and decidual caps from each litter washed, pooled, weighed, and counted. The results were tabulated as cpm per gram of tissue, and the means and standard deviations calculated for each group. Student's T-test (two-tailed) was used to assess the significance of the difference between group means.

The binding of 20-8 to control decidual tissue was quite variable but seemed to decrease as the litter size increased (Table 6). The binding of 20-8 to target decidua was at least twice as much as control values. The control decidua bound 28-8 more consistently with no dependence on litter size. Again, the binding to target decidua was about twice control values. Both control and target yolk sac seemed to concentrate either antibody nonspecifically since consistently high levels of binding were observed which was irrespective of the embryonic genotype. Although the background (control)

binding to placenta was relatively high (16,900 cpm/g for 20-8 and 12,900 cpm/g for 28-8), both antibodies bound 2.5 times higher to target placentas. About two times more 20-8 antibody leaked into the control fetus than the target fetus. However, the 28-8 antibody binding was significiantly different in target versus control fetuses ($P < 0.02$), the target fetuses containing somewhat higher counts. Since the overall level of radioactivity in fetal tissue was so low, the comparatively low specific activity of the 28-8 antibody could have contributed to these effects. Thus, there was no dramatic difference in the absorption pattern of any gestational tissues between the broadly reactive or specific anti-class I antibody.

D. Preparation of Cell Suspensions From Placental Tissue.

Previous studies were inconclusive in localizing and characterizing class I antigens in gestational tissues. It was apparent that these proteins should be isolated in order to be identified. Placentas, yolk sacs, and fetal livers from day 16 of gestation were dissected, minced, pushed through a fine mesh screen and washed. The resulting cell suspensions were microscopically evaluated. Placentas routinely yielded approximately 5×10^7 cells apiece. 75% of these cells were either fetal or adult red blood cells (identified by size, shape, and thickness of the plasma membrane) and their percentage did not decrease if the maternal circulation was first flushed with heparinized PBS. Hypotonic shock did not change the ratio of the cell types. The remaining 25% of the original population consisted of large and irregularly shaped, and about 80% dead (as assessed by trypan blue exclusion). Total cell yields from yolk sac varied but were between $5 - 10 \times 10^6$ per tissue. 75% of these cells were the round, thick-walled erythrocytes and 25% were large, granular, irregularly shaped cells, almost all dead. Hypotonic shock removed only half of the red cells, presumably because immature fetal reticulocytes are more resistant to lysis. Day 16 liver yielded about $3\text{-}4 \times 10^7$ cells, only 25% of which were red cells. These erythrocytes were all sensitive to hypotonic shock. The remaining $1\text{-}3 \times 10^7$ cells were live liver cells of various sizes and shapes. These irregular cells were also sensitive to osmotic stress as hypotonic shock destroyed about 75% of them.

Due to the high level of cell death encountered in mechanical disruption of the tissue, we attempted collagenase digestion as a way of preparing

suspensions of live cells. The number of cells obtained was generally lower than the number obtained from mechanical disruption, but cell viabilities were much increased.

The cells from collagenase digested tissues were labeled with ^{125}I using the iodogen method (Mishell and Shigii, 1980) and solubilized membranes prepared. Since very little radioactivity (2-6 x 10^7 cpm/10^8 cells) was incorporated into the solubilized membrane preparations (or the spleen cell controls), the enzyme treated cell preparations were checked for the presence of membrane bound H-2 antigens via indirect immunofluorescence. Monoclonal antibody bound to greater than 95% of spleen or liver cells prepared by mechanical disruption but did not bind at all to collagenase treated cells. It is worth noting here that the collagenase preparation which worked best to free cells from their tissue matrix was a crude preparation, undoubtedly contaminated with proteases. Thus, the enzyme treatment, although mild enough to retain viability, stripped the cells of much of their surface protein.

E. Analysis of Class I specific RNA

The few Qa and Tla antigens that have been isolated using immunoprecipitation with defined antibodies resembled the traditional class I antigens biochemically (Rothenberg and Triglia, 1981; Michaelson, *et al.*, 1982, 1983; McIntyre, *et al*, 1982 and Yokoyama, *et al.*, 1981, 1982). These unusual antigens, however, are not as polymorphic or widespread in their expressionas the traditional class I antigens, and thus difficult to identify. In the early 1980's using a molecular probe derived from the DNA encoding the most conserved portion of the class I molecules, the C2 domain, Hood's laboratory identified at least 30 class I-like genes in a cosmid library of BALB/c sperm (genomic) DNA. All of these genes (or gene pieces) mapped to the MHC on chromosome 17 -- no other DNA hybridized to the probe, even at low stringency (Steinmetz, *et al.*, 1981 and Winoto, *et al.*, 1983) Thus, it appeared that although only a few genes were commonly expressed, many more genes were available for expression, given the correct transcription and translation signals. Goodenow and colleagues (1982) transfected DNA from the cosmid library into L-cells, stained the transfectants with a bank of monoclonal antibodies specific for class I antigens, and thus matched some of the genes with their products. The K, D, and L genes were located, and also the gene encoding the secreted Q10

(Q10^d), and, subsequently, TL (T3/T13^d) (Fisher, Hunt, and Hood, 1985). Similar studies done by Flavell's group using a different strain (Lew, *et al.*, 1986a; Mellor, 1986 and Flavell, *et al.*, 1986) revealed a similar picture, although in the B10 mouse the D gene is allelic to the BALB/c L gene (and there is no gene at the L locus), the Qa region shows some gene duplication and deletions, and the number of genes in the Tla region is only about half that of the BALB Tla region. These investigators identified Q8^b as the gene encoding the serologically detectable Qa-2^a antigen. Qa-2^a is encoded by D2^d in the BALB/c mouse (Hunt, unpublished observations). Thus, it was clear that the search for novel class I specificities should be confined to a search of the MHC.

Based upon the foregoing results, and the work of Gill and colleagues with the Pa antigen (see "Introduction"), we reasoned that non-traditional class I antigens were present in gestational tissues, and the these determinants might be encoded by the non-traditional class I genes.

Twenty-two oligonucleotide probes identifying 29 of the 33 genes in the BALB/c MHC were synthesized by S. Hunt in the laboratory of L. Hood (California Institute of Technology, Pasadena, CA) and kindly provided to us as soluble preparations (Table 2). It should be noted that subsequent to the generation of the probes, the molecular map of the BALB/c MHC was refined. Consequently, the designations and location of the corresponding genes was changed to more closely agree to that established in other haplotypes. Thus, the original probe designations are used throughout this text with reference to the current gene nomenclature. These probes were all derived from the putative transmembrane region of the genes (as recognized by homology to known class I genes and predicted amino acid sequences). This area of each gene was found to be one of the most variable regions, and was thus a good candidate for the construction of synthetic oligonucleotide probes able to distinguish the genes horizontally (within the BALB genome). The specific probes had few to no cross hybridizations to genes within the BALB cosmid library, and only Pr11-2 cross hybridized to rRNA. The degree of homology (predictive of cross-hybridization) to the MHC genes of other strains is largely unknown (Table 2).

1. Dot Blots.

Preliminary studies were performed using formaldehyde fixed cell lysates from C3H x C3H (control) or C3H x BALB (target) gestational tissues dot

blotted onto nitrocellulose. Although the overall binding of probes PrD, PrQ1, PrQ8, PrQ10, and PrT13 differed, there was no discrimination between target or control binding (Figure 9). Therefore, B10 x B10 (control) and B10 x BALB (target) matings were used. Again, the level of hybridization varied more with the type of tissue than with the probes used (PrD, PrL, PrQ1, PrQ7, PrQ8, PrQ10, PrT4, and PrT13). Thus, the highest intensity of binding was on late gestation (day 16) liver, with moderate labeling seen on late gestation spleen and thymus, and some labeling of placenta, fetal membranes and post-implantation (day 7) embryo. This pattern was indicative of the non-specific binding frequently observed in samples with significant degradation of the RNA. This is common in tissues such as liver and pancreas that have high concentrations of endogenous nucleases and also of tissues subject to increased cell death as a result of the extended time needed to dissect small, relatively inaccessible fetal organs such as thymus and spleen. This approach was then abandoned and purer RNA preparations obtained by phenol:chloroform extraction of the proteins. Degraded samples can be identified and eliminated from the analysis based upon the rRNA band separation seen in material electrophoresed in agarose gels. This process, however, requires larger amounts of tissue and so the immediate postimplantation period could not be surveyed.

2. Northern Blots.

Hybridizations were always performed in groups so that a few probes were exposed to the filters under seperate but identical conditions. This enabled us to include an internal control (a probe hybridizing to a gene known to be expressed) in each hybridization.

The classical H-2 genes, K, D and L were expressed in day 11 through day 16 placenta, days 11, 12, 13 and 15 yolk sac, and days 13-16 fetal liver (Table 7, Figure 9.). PrK (which identifies the H-2K^d gene) bound equally well to RNA from both B10 x BALB and B10 x B10 matings confirming a cross reactivity of this probe with a different gene. Cross reaction of PrK to a TI region gene was observed when PrK was hybridized to a B6 cosmid library (Hunt, unpublished observations). The PrD probe (H-2D^d) hybridized to RNA from day 10-16 B10 x BALB placenta but not B10 x B10 tissue. The H-2D^d message was detected in B10 x BALB placenta (but not B10 x B10) and day 11 and 12 yolk sac and absent from yolk sac on days 13 through 16. There was no binding of

PrD to fetal liver RNA. PrL (specific for H-2L^d) hybridized specifically to day 13-16 B10 x BALB placenta and was not detected in day 11 or day 12 placenta, day 11, 12, or 15 yolk sac, or day 13-16 fetal liver.

The K2 gene which is just centromeric to H-2K^d is detected by the Pr11-2 probe. This probe bound strongly to all RNA samples even after a 60^{o}C. wash. Pr2-2 which detects the D/L region gene centromeric to L (D4^d) hybridized weakly to all samples but washed off at 50^o C. Two genes, D2^d and D3^d, thought to lie in the Qa subregion were formerly identified as Q9 and Q10 respectively. Pr10 identifies D2 and did not hybridize to the gestational tissue RNA. There was no probe for D3.

The melting temperature (T_m) is a measure of the thermal stability of DNA-DNA or DNA-RNA hybrids. Under given conditions of temperature, ionic strength, and concentrations of denaturants (i.e. stringency), the temperature at which a molecular probe of a given length will wash away from the nucleic acid fixed to a nitrocellulose sheet can be calculated. The T_m for the probes that did not survive the 50^o C. wash were: Pr2-2, 53.7^o; PrT3/13, 54.3^o and PrT10/18, 58.2^o. (T_m = 69.3 + 0.41(GC%) - 650/L where the GC% is the percent of guanine and cytosine and L is the length (in base pairs) of the probe (Maniatis, Fritsch and Sambrook, 1982).) The 50^o wash was close to the melting temperatures of Pr2-2 and PrT3/13, and so the binding of these probes at the lower stringency wash was probably real. By the same criteria, the PrT10/18 binding only at low stringency casts doubt on the expression of the T10 or T18 genes.

None of the Qa region probes bound to gestational tissue RNA, even at low stringency. Thus, it appears that Q1, Q4, Q6 , Q7 (27.1), Q8/9, or Q10 are not expressed during mid and late murine gestation. There was no probe available for Q2 and the PrD probe also detects Q5, so the expression of these genes cannot yet be determined.

Probes detecting the T2, T4, T5, T6, T8, T9, T12, T14, T16 and T17 genes did not hybridize. Pr T1/11 (detects both the T1 and T11 genes) hybridized to day 12-16 placenta (but not day 11 placenta), days 11, 12, 14, and 16 yolk sac, and day 13-16 fetal liver from either mating combination. Although this binding was weak, it did persist through the higher stringency (50^o C.) wash. Similarly, PrT7/T15 bound equally to all samples tested, and this binding persisted through a 60^o C. wash. PrT3/T13, which detects the gene encoding the serologically detectable TL antigen, bound to all the RNA samples but the

binding did not survive a high stringency (50° C. wash). PrT10 also bound at low stringency and washed off under higher stringency.

The overall conclusions that arise from these molecular studies are: 1) the classical H-2 genes (K, D and L), as well as the neighboring genes that are situated in the same orientation, are expressed in murine mid-gestational tissues; 2) the two genes slightly distal to L, whose reading frame is opposite to D and L, named D2 and D3, are not expressed in detectable amounts; 3) none of the probes recognizing Qa region genes bound to total RNA of gestational tissues; 4) four of the TI region probes were positive indicating the expression of as many as 8 (of 18) genes. There is yet no obvious link between these expressed genes and no molecular reason for their expression as opposed to any other TI gene.

Discussion

Active anti-paternal transplantation immunity and pregnancy can co-exist in the same female. The interface at which these antagonists meet is the trophoblast. Trophoblast tissue is found mostly in the placenta with some minor populations of trophoblast cells interpolating between fetal membranes and maternal decidua/uterus. Thus, much of the interaction between the feto-placental unit and immune effectors will occur at or in the placenta, a complex organ which varies tremendously across species in its ontogeny, structure and architecture. Beer and Billingham (1976) put it beautifully: the placenta "must temporarily serve as a fetal lung, kidney, intestine, and liver as well as a complex endocrine organ that completely takes over the functions of the maternal ovary and pituitary and initiates and completes complex endocrine functions requiring input from both the mother and her immature fetus. In addition, the human (and rodent) placenta transmits to the fetus a dowry of prophylactic antibodies as it develops in a sterile environment, as if anticipating its sudden need of protection on exposure to the contaminated external environment at the time of parturition. Nonetheless this relationship is always finite."

Maternal and fetal circulations do not mix within the placenta, although the fetal trophoblast invades maternal tissue to different degrees in different species. In the extreme case, the fetal villi penetrate through uterine epithelium, stroma and spiral artery endothelium, thus exposing the trophoblast directly to maternal blood. This hemochorial type of placentation exists in both the rodents and primates although there are ultrastructural differences in the two groups.

The events in immunological rejection of grafted tissue are less variable among mammals. Rejection of tissue and/or organ grafts occur in reproducible patterns that are chiefly dependent upon MHC antigenic disparity, and does not differ significantly from species to species (Goetz, 1977). Indeed, skin grafts that are MHC disparate from donor to host are rejected faster than grafts across species lines (Lafferty and Jones, 1969). In addition, proliferation of cells in the MLR is much greater in response to an allogeneic rather than a xenogeneic stimulus (Wilson and Nowell, 1970 and Widmer and Bach, 1972). Thus, although the cellular mechanisms are still uncertain (Lafferty, *et al.*,1983 and

Mason and Morris, 1986), the morphology and kinetics of graft rejection are similar and predictable.

The role of the trophoblast in preventing immune rejection of the fetus has been convincingly demonstrated by Rossant and her colleagues (Rossant, *et al.*, 1983). *Mus caroli* (an Asian field mouse) does not interbreed with *Mus musculus* (the standard laboratory "house mouse"), and caroli blastocysts transferred to musculus uteri do not survive (Frels, *et al.*, 1980). The embryos are resorbed around day 10-11 and maternal T-cells that kill *M. caroli* targets in a standard CTL assay can be obtained from the failed implantation sites (Croy, Rossant and Clark, 1982). There is histological evidence of lymphocyte infiltration into day 9.5 implantations, prior to any morphological evidence of embryonic distress, and no cytotoxic cells were found in successful allogeneic pregnancies even if they inhabited the same uterus as the dying *M. caroli* embryos. *M. caroli M. musculus* embryos, made by microinjection of the caroli ICM into the musculus blastocyst (Rossant and Frels, 1980), undergo normal gestation in the *M. musculus* uterus and produce healthy chimeras. The reciprocal experiment, musculus ICMs placed into the caroli blastscysts and transfered to a pseudopregnant *M. musculus* produced no offspring. Indeed, a reconstituted blastocyst composed of a *M. caroli* ICM totally surrounded by *M. musculus* trophectoderm will implant and develop to term in the musculus uterus producing a normal *M. caroli* offspring (Rossant, *et al.*, 1983). A similar model has been developed in farm animals in which "shoats"--chimeras made by fusing goat and sheep embryos before transfer avoids the apparent immunological rejection of a goat embryo by a sheep uterus (Fehilly, *et al.*, 1984 and Meinecke-Tillman and Meinecke, 1984).

We are faced with a paradox. While histoincompatibility induces the strongest graft rejection, it is interspecies pregnancy, and not allogeneic pregnancy, which is apparently immunologically rejected. However, recent observations by Croy and colleagues (Rossant and Croy, 1987 and unpublished observations) have placed doubt upon this hypothesis: caroli blastocysts die on schedule when transferred to an immunodeficient (nude or SCID) musculus uterus. In addition, immunological reactions are not always destructive. Activated T-cells produce a plethora of lymphokines that induce growth and differentiation in resting cells (deWeck, 1984). Ongoing immune responses can augment wound healing (Green and Wegmann, 1987) and tumor growth (Prehn, 1983), and the cytokines that stimulate cells of the

macrophage lineage produce ten-fold increases in the growth of placental cells *in vitro* (Athanassakis, *et al.*, 1987). Indeed, treatment of the pregnant female with anti-thymocyte serum or monoclonal anti-Lyt 2 antibody decreases this growth surge by a half, and obviates the macrophage-like functions of the cultured placental cells. Outbred and allogeneic matings produce larger sized fetuses, but the immunological basis of this is questionable (McLaren, 1975), although at least one study shows that active immunization promotes larger feto-placental units (Beer, Scott and Billingham, 1975).

The trophoblast is thus invariably associated with the modulation of maternal immunity. Trophoblast provides both the immunogens for immunostimulation and a barrier to any cytotoxic effectors that might be generated in the course of lymphokine production. The work summarized in this thesis helped define this complex tissue in the context of its immunologic function(s).

The first issue addressed was the efficiency of the barrier to transplacental cellular traffic. Using electrophoretic analysis of GPI isozymes, we found no evidence of maternal to fetal passage of cells in the vast majority of animals assayed. Maternal cells were detected in only two of 172 offspring tested, and both of these animals contained a relatively high percentage of transplacental migrants. In our study, each animal was sacrificed in order to assess trafficking. Therefore, we could not follow the development of neonates containing maternal cells. It has been suggested, but not confirmed, that passage of lymphocytes from mother to neonate during suckling could be the cause of the runting seen in the offspring of some allogeneic matings (Beer and Billingham, 1973; Beer, Billingham, and Head, 1975; but see also Silvers and Poole, 1975). If this is true, by extension then, a massive infusion of lymphocytes across the placenta could cause significant immunologic effects. Once released from the immunosuppressive effects of the fetal environment, these cells might easily mount a graft-versus-host response that could lead to runting (Beer and Billingham, 1973). One could speculate that the two animals in which we found maternal cells provide one explanation for the occasional runt in an otherwise healthy litter. Indeed, there is convincing evidence for the transplacental passage of lymphocytes into human fetuses in children with severe combined immunodeficiency disease whose graft-versus-host-syndrome is associated with maternal immunocompetent cells.(Kadowaki, *et al.*, 1965; Turner, *et al.*, 1966; Githens, *et al.*, 1969; O'Reilley, *et al.*, 1973; Pollack,

et al., 1980; 1982; Rosenstock, *et al.*, 1981; Flomenberg, *et al.*, 1983 and Geha and Reinhertz, 1985). These results contrast with many previous studies showing maternal to fetal trafficking. Detection of fluorochrome-labeled cells (Desai and Creger, 1963), karyotype analysis of morphologically distinct chromosomal markers (Tuffrey, Bishun, and Barnes, 1969 a, b and Schroder, 1974) , and autoradiography of radioactively labeled cells (Barnes and Holliday, 1970 and Barnes and Tuffrey, 1970) have all supported the notion that the fetus normally and regularly receives maternal cell transfusions from across the placenta. Others, however, using similar techniques report no such trafficking (Feingold and Mitchie, 1961; Billington, *et al.*, 1969; Silier, 1970; Schroder and Anderson, 1975 and Philip, *et al.*, 1982). Interpretation of such studies is problematical since the markers are either easily exchanged between cells, or difficult to recognize unequivocally. Skepticism must be used in evaluating results of postpartum examination of offspring since circulatory exchange at parturition and the cellular contributions due to suckling are not usually considered.

Collins, Chrest and Adler (1984) reported extensive trafficking into the murine fetus. These authors used allo-specific antisera and indirect immunofluorescence to examine the liver cells of 17 day fetuses that had been transplanted as blastocysts to allogeneic foster mothers. Supposed maternal to fetal transplacental migration of cells occurred in as many as 92% of the offspring in a litter. The allogeneic cells usually comprised 12-40% of the fetal liver, although contributions from allogeneic embryos occupying the same litter could not be eliminated as a source of cells. Furthermore, 8% of the fetuses did not react at all with antisera specific for their own MHC phenotype, but were apparently reconstituted by maternal cells. It is difficult to interpret these results since artificial mixtures were not included in the immunofluorescence controls.

Electrophoretic resolution of GPI isozymes provides a direct and obvious distinction between maternal and fetal cells. One need not depend on the binding affinity or cross reactivities of antibodies, morphologic segregation of chromosomes, or other techniques subject to background effects. Using this method, we were unable to detect trafficking in most animals, and thus could not verify the above claims. The only practical limitation of this system is its sensitivity. With a resolution limit of 1%, as many as 10^6 blood cells or 10^5 spleen or liver cells of maternal origin could escape detection.

The FACS experiments were designed to increase the sensitivity compared to the electrophoretic system. Extensive studies by Butcher and colleagues (Butcher and Weissman, 1980 and Butcher, Scollay, and Weissman, 1980) showed that FITC is concentrated in the cytoplasm of labeled cells, and that membrane functions such as homing and antigen recognition are not altered by fluorescent labeling. In general, our FACS studies supported the GPI analyses in that we found scant evidence of WBC trafficking. However, small numbers of labeled RBC appeared to cross the placenta since generally 0.40% of the intensely labeled cells remaining in maternal circulation after 24 hours were detectable in the fetal blood. Occasionally, trafficking was observed to a greater extent since 10% of the neonates contained large numbers of labeled cells. This indicates that RBC can cross the placenta, sometimes with great ease, and that RBC trafficking can vary dramatically from one fetus to another. Erythrocytes are well-suited to transplacental migration because their size and membrane architecture make them particularly elastic, and they are deficient in many cell surface interaction molecules. Indeed, in most species class II MHC antigens are absent, and class I MHC antigens are expressed in only low levels on murine RBCs (Goetze, 1977).

The tremendous proliferative capacity of lymphocytes in response to antigen might make transplacental transport of even a few sensitized cells a dangerous event. In one of possibly two cases we observed significant but low levels of labeled WSC in the fetal liver. Considering all the animals, the average number of WSC appearing in the fetal liver was 225 (range 0 - 3100). This number was obtained by multiplying the number of cells recovered from the liver (5×10^6 cells) by the percent of intensely labeled WSC detected (0.0044%). Clearly then, according to FACS analysis, WSC trafficking is very low or nonexistent in almost all fetuses Within the limits of the techniques used, it is clear that significant maternal to fetal migration of WBC is a rare event. The placenta provides a barrier to the passage of WSC, but not RBC, and that barrier is very efficient, being maintained throughout the later stages of gestation.

Sensitized maternal anti-fetal effector cells may be able to overcome this block. We intended to investigate this possibliity by using BALB female mice intraperitonealy primed ten days prior by 10^7 C3H splenocytes as the source of FITC-labeled WSC. Limiting dilution analysis of cells from naive versus primed BALB mice however, showed that the precursor frequency of proliferating cells

increases only three-fold (1:1200 to 1:400, respectively). This was insufficient enrichment to observe real differences in the potentially trafficking population and so this avenue was not pursued. Beer and Billingham (1973) observed that the adoptive transfer of maternal lymphocytes primed to paternal alloantigens led to a high incidence of runt disease in the litters even though the babies appeared normal at delivery. However, the offspring were not fostered and so the possibility that these effects were due to the acquisition of sensitized cells during suckling was not eliminated. Preliminary studies reported in the literature claim that females can undergo vigorous alloimmune responses while gestating healthy embryos bearing target antigens. These studies provide circumstantial evidence that immune effectors do not enter the fetus (Mitchinson, 1953; Woodruff, 1958; Lanman, *et al.*, 1962; Beer and Billingham, 1973 and Wegmann, *et al.*, 1979b).

The trafficking studies established that the placenta can act at least as an anatomical barrier to white blood cells. Previous work in this laboratory (Wegmann, Singh and Carlson, 1979; Wegmann, *et al.*, 1979 and Singh, *et al.*, 1983) and others (Bell and Billington, 1983a; Chatterjee-Hasuroni and Lala, 1982 and Adeniyi-Jones and Ozato, 1987) has shown that the placenta and/or fetal membranes can specifically absorb antibodies of anti-paternal specificity from maternal circulation. The density of MHC antigens is low, H-2K^k is present at about 1.1 x 10^{13} molecules/gram of placenta, or 10^4 molecules/cell assuming uniform distribution (Ragupathy, *et al.*, 1981) while lymphocytes have about 10^5 H-2K molecules/cell. In addition, saturation of the target sites for placental absorption of maternal anti-paternal antibody is achieved at relatively low titers (1:128, Bell and Billington, 1983c) although the specificity of the antibodies in the study was not determined. Adeniyi-Jones and Ozato (1987) found that although the placenta specifically absorbed monoclonal anti-paternal class I antibody from maternal circulation, spillover into the fetus occurred at low doses. The ascites-derived antibody they used, however, was not absorbed/eluted on the target epitopes, and could thus contain significant amounts of antibody not directed towards paternal alloantigens. Such antibodies would be passed through the yolk sac via Fc receptor binding and transport and accumulate in the fetus. Thus, it became important to identify and characterize the appropriate antigens that removed antibodies and possibly also removed activated cells from the immediate vicinity of the fetus. Since mature trophoblast is deficient in MHC antigen expression, we decided to

reinvestigate the question of H-2 ontogeny in gestational tissues using the increased sensitivity and specificity available with monoclonal antibodies.

Several NIH typing sera that had been absorbed to limited specificity were used in an attempt to confirm previous findings showing the presence of MHC class I antigens on murine oocytes, and the disappearance of these antigens during the preimplantation period of development (Heyner, Brinster, and Palm, 1971; Palm, Heyner and Brinster, 1971; Gardner, Johnson and Edwards, 1973; Searle, *et al.*, 1974; Hakansson, *et al.*, 1975; Muggleton-Harris and Johnson, 1976; Searle, *et al.*, 1976; Billington, *et al.*, 1977; Jenkinson and Billington, 1977; Krco and Goldberg, 1977; Webb, *et al.*, 1977 and Heyner and Hunziker, 1979). H-2^b, H-2^d or H-2^k metaphase oocytes did not bind to any of the appropriate antisera as detected by indirect immunofluorescence under conditions that gave optimal binding of splenic WBC. Heyner and Hunziker (1979, 1981) reported H-2 class I antigen expression which decreased to undetectable levels after fertilization. The antisera used in the studies of Heyner and Hunziker, however, were different from the reagents used in the work presented in this thesis. In light of current theories about cross reactivities of anti-class I antibodies amoung the entire MHC gene family (Cook, *et al.*, 1983; Figueora, *et al.*, 1983 and Sharrow, Flaherty, and Sachs, 1984), these differences in the source of the reagents used could be significant.

Anti-H-2^d antiserum bound to BALB blastocysts but also (to a lesser degree) to C3H blastocysts. The antibody did not react with B10 blastocysts, nor to the trophoblast cells of blastocyst implants. However, the emerging endodermal cells overlying the ICM bound antibody equally well regardless of strain. This might not be surprising in light of the fact that yolk sac, the terminally differentiated derivative of endoderm, has an extensive number of Fc receptors. Anti-H-2^k antiserum bound strongly to C3H blastocysts, but was absent from the trophoblast of blastocyst implants. There were no endoderm cells in these cultures. Previous investigators have reported negative, weak, or positive binding of anti-class I antisera to either the blastocyst trophectoderm, ICM, or both (Heyner, Brinster and Palm, 1971; Palm, Heyner and Brinster, 1971; Gardner, Johnson and Edwards, 1973; Heyner, 1973; Searle, *et al*, 1974; Hakansson, *et al.*, 1975; Muggleton-Harris and Johnson, 1976; Searle, *et al.*, 1976; Jenkinson and Billington, 1977; Billington, *et al.*, 1977; Sellens, 1977; Webb, *et al.*, 1977 and Heyner and Hunziker, 1979). None of four different monoclonal antibodies specific for three different class I molecules reacted with

oocytes or blastocysts. This result agrees with the previous findings that monoclonal anti-H-2K^k antibodies, even in cocktail and used at various dilutions and binding temperatures, do not bind to mouse oocytes (Heyner and Hunziker, 1981 and unpublished results). Lala, Kearns, and Colavincenzo (1984) however showed low levels of transient binding of anti H-2K^k to morulae, and Warner's group (Warner and Spannaus, 1984 and Goldbard, *et al.*, 1985) has shown weak binding to blastocysts of a rat anti-mouse monoclonal antibody that recognizes the conserved alpha$_3$ domain of all class I molecules. These antigens are no longer detectable by the onset of implantation (Hakansson, *et al.*, 1975; Hakansson and Sunqvist, 1975: Searle, *et al.*, 1976; Billington, *et al.*, 1977 and Leclipteux and Remacle, 1983), an observation that led Sellens, *et al.* (1978) to speculate that this provided an escape from maternal immune recognition. Since the mature placenta and the mid-gestational fetus express class I antigens, this delaying of the onset of a potentially destructive maternal immune response until after the formation of a barrier could be an important mechanism to insure fetal survival.

In our studies, one of two monoclonal antibodies reactive with beta$_2$ microglobulin bound to the appropriate blastocyst (but not the corresponding oocyte). Unfortunately, the S19 ascites that showed this interesting result was unavailable in quanitity so we could not confirm its specificity by RIA in our laboratory, although indirect immunofluorescence on spleen cells confirmed its reactivity with B10 but not C3H or BALB spleen cells.

Nevertheless, these results were provocative and suggested the presence of non traditional class I antigens on the earliest stages of trophoblast differentiation. Beta$_2$ microglobulin is invariably found in association with class I antigens at the cell surface, and its lack is associated with a low cell surface expression of class I antigens (Goodenow, *et al.*, 1982 and Winoto, *et al.*, 1983). The possible presence of beta$_2$ microglobulin on blastocysts in the absence of reactivity of polymorphic anti-class I antibodies indicated the presence of non-classical class I antigens (i.e. those encoded by the Qa or Tla loci of the MHC) on trophectoderm. Beta$_2$ microglobulin does not have a transmembrane cytoplasmic tail and so must be associated with a membrane-bound molecule in order to be detected at the cell surface. The Qa antigens can act as transplantation antigens and generate both antobody and CTL responses (Flaherty, 1981), although a limited polymorphism and tissue distribution may make these molecules poor immunogens. Although both traditional and non-

traditional class I antigens have been found on the rat placenta (Macpherson, *et al.*, 1987 and Ho, *et al.*, 1987), only the non-traditional Pa antigen ellicits an antibody response. Ho and colleagues have speculated that this observation suggests that only the non-traditional antigens are exposed to maternal tissues, the traditional determinants being sequestered on the fetal side of the placenta. Thus, the complement-binding IgG_2 isotype antibody characteristic of an antibody response towards traditional class I determinants is not produced, and a non-destructive IgG_1 response with specificity for the non-traditional antigen is seen.

Most of the class I genes in the murine MHC are located in the Qa and Tla regions of the complex (Figure 6), telomeric to the D/L region. The cross-strain polymorphism and tissue distribution of the gene products is very limited compared to the more traditional genes of the K, D and L loci. TL antigens were first discovered by Old, *et al.* (1963) as thymocyte cross-reactive determinants in attempts to raise anti-leukemia sera. Linkage studies mapped the locus controlling expression of this antigen near the MHC telomeric to the H-2D locus. Reactivity of the absorbed antisera on various strains indicated complex patterns, and thus six alleles were assigned to the new locus (Old and Stockert, 1977; Flaherty, 1981; and Shen, *et al.*, 1982). The interpretation of these results is not conclusive since it is now apparent that the Tla region of the chromosome contains more genes than any other (Figure 6), and it is not known how many of these genes contribute to the haplotypes expressed. TL antigens are found only in the thymus of normal mice, although proliferating T-cells re-express TL (Cook and Landolfini, 1983). There appears to be a maximum amount of class I antigen that can be expressed on the cell surface since H-2D antigen expression is reduced in TL positive strains (Flaherty, 1981).

The Qa antigens were discovered when some unusual tissue reactivity patterns emerged from assays of anti-TL sera. The binding of absorbed antisera to a subpopulation of lymphocytes (from lymph node and spleen) indicated an additional reactivity that was labeled "Qa-1". Qa-1 is present in the lymph node, spleen, [illegible] and bone marrow, and appears to be a marker for certain "inducer" a[illegible]plifier" T-cells, having been especially well characterized in suppres[illegible] networks (Lynes, *et al.*, 1982; Green, *et al.*, 1983). The Qa-2 antigen was defined by the cytotoxicity reactions of two congenic lines to antibodies prepared in one of the lines to the parent strain. Qa-2 is also found on subsets of T-cells, but contrary to TL, is largely [illegible] from

thymocytes. Monoclonal antibodies have helped further define the tissue distribution and biochemistry of Qa-1 and Qa-2 as well as identifying many additional specificities controlled by genes in this region (Sutton, V. R., *et al.*, 1983; Michaelson, *et al.*, 1977; Sandrin, *et al.*, 1983; Hogarth, *et al.*, 1982 and Hammerling, *et al.*, 1979).

With the possible exception of Qa-1, most of the genes encoding these antigens apparently possesses only two alleles, one which encodes the Qa determinant, and a null, non-functional allele (Michaelson, *et al.*, 1982; 1983; Flaherty, *et al.*, 1985 and Lew, Maloy and Colligan, 1987). A few of these non-traditional antigens have been isolated and characterized. Qa-1 (Rothenberg and Triglia, 1981) and Qa-2 (Michaelson, *et al.*, 1982, 1983), and TL (McIntyre, *et al.*, 1982 and Yokoyama, *et al.*, 1981 and 1982) conform to all biochemical criteria for class I genes: heavy chain (molecular weight approximately 40-45 kd) of three external domains with a transmembrane portion and cytoplasmic tail in non-covalent association with beta2 microglobulin. Q10 is also a typical class I antigen except that a truncated TM region allows its expression as a secreted molecule (Mellor, *et al.*, 1984).

We tested the possibility that Qa/Tla antigens were present in gestational tissues by examining the immunoabsorbant capacity of mature placenta for two different anti-class I antibodies. 28-8-6S is an IgG_{2a} monoclonal antibody derived from the fusion of spleen cells (from C3H mice immunized against C3H.SW splenocytes) and the hybridoma variant SP2/0Ag.14 (Ozato and Sachs, 1981). Ascites fluid from BALB/c mice bearing this tumor was very restrictive in its activity, binding only to cells expressing $H\text{-}2K^b$ or $H\text{-}2D^b$. The monoclonal antibody 20-8-4S was raised in an identical fashion. Yet this IgG_{2a} antibody, originally characterized as anti-$H\text{-}2K^b$ $H\text{-}2D^b$, $H\text{-}2^r$, $H\text{-}2^s$ also reacts with lymph nodes and spleen of $H\text{-}2^a$ and $H\text{-}2^q$, but not $H\text{-}2^k$, $H\text{-}2^p$ or $H\text{-}2^f$ (Sharrow, Flaherty, and Sachs, 1984). The cross reactive antigen was mapped to the Qa region by indirect immunofluorescence assay of various congenic recombinant strains. Both of these antibodies were protein A purified from ascites fluid, ^{125}I-labeled, and absorbed, then eluted from target (B10) cells. The specific activity of the absorbed/eluted antibodies was defined as the percent of cpm added that bound to target (as compared to control) cells. The specific binding to B10 was quite variable: different preparations of 20-8 were 60-75% specific while the specificity of 28-8 ranged from 27-45%. We attributed the individual variation to different degrees of denaturation caused by the iodine

coupling procedure, and the overall lower binding of 28-8 to either the presence of fewer target antigens/cell, or, more likely, a lower binding affinity. Nevertheless, we attempted to equalize these differences by administering roughly equivalent numbers of specific cpm per mouse.

Mothers on day 15 of pregnancy were chosen since previous work showed that placental absorbance of ^{125}I-labeled anti-class I antibodies was maximal at that point (Ragupathy, *et al.*, 1981). The antibodies were allowed six hours to reach equilibrium binding with little degradation (Ragupathy, *et al.*, 1984). Within the rather crude limits of the system, it was clear that there were no dramatic differences between the immunoabsorbence of the two antibodies. Target decidual caps bound as many cpm as control decidual caps. This seems unusual since the decidua is almost entirely of maternal GPI isotype (Rossant and Croy, 1985) and thus essentially free of paternal (target) epitopes. It is possible that the overall smaller litter size in the target matings could contribute to this effect, especially in the absorbance of 20-8, which was clearly increased as the number of pups decreased.

Yolk sac is apparently a sink for antibody since some of the highest binding was found in either target or control yolk sac. This is not unexpected since the yolk sac is one of the major routes of passive Ig transport to the fetus (Laliberte, 1985), and most of this uptake is mediated by Fc receptor binding.

The binding to target placentas was 2.5 times that of control values for both antibodies. This is evidence for the existence of approximately equal amounts of the target epitopes specific for each antibody. Had there been an additional non-traditional class I antigen expressed in the placenta, the 20-8 antibody should have bound to a significantly greater extent. It is worth noting that in all cases, the cpm bound in these studies was in excess of that observed by previous workers. Ragupathy, *et al.*, (1979, 1981) reported target placentas binding between 1000-1500 cpm per gram to tissue while controls bound 300-700 cpm per gram of tissue. Fetuses contained between 200-600 cpm/g. The monoclonal antibody used in those studies was a broadly reactive anti-H-2K^k (65% specific activity) and 2-5 x 10^5 cpm were administered to each mother on days 10, 13 or 17 of gestation. Other procedural differences involved the preparation of single placental cell suspensions prior to counting of the gamma radiation. The gestational tissues in the work reported here were simply washed thoroughly before counting. Nevertheless, the binding of antibody to

target placentas in the studies of Ragupathy and colleagues was 2-3 times higher than controls, so the ratios in all of these studies were similar.

The only real difference between 20-8 and 28-8 in their binding to gestational tissues was observed in the amount of antibody that spilled over into the fetus. As has been reported for other broadly reactive anti-H-2 antibodies, H-2K^k and H-2D^k (Ragupathy, *et al.*, 1981), the target fetuses contained only about half the cpm of the controls. Thus, the placenta blocks the free exchange of antibody between mother and fetus. The 28-8 reagent, however, showed approximately equivalent amounts of antibody in either control or target fetuses. It is difficult to draw conclusions from these values, since the standard deviations are large, the sample sizes small, and the cpm detected such a small percentage of the actual number of cpm injected. It must be noted that the specific activity of 28-8 was, at best, only two-thirds that of 20-8, thus the mice receiving 28-8 had a larger radioactivity burden and possibly higher backgrounds, since more total counts of 28-8 were injected into the mothers.

Although there were no differences in relative binding of the two antibodies, this could be due to the presence of the elusive antigens on such a small percentage of placental cells that experiments based on binding capacity of the entire organ were too insensitive to reveal differences. It was clear that more sensitive and direct techniques would be required to reveal any non-traditional class I determinants. These conclusions led to efforts to prepare healthy, clean cells in large numbers from gestational tissues in anticipation of radioiodination and immunoprecipitation of the class I antigens. We believed that monoclonal anti-beta2 microglobulin would coprecipitate all the class I heavy chains with which beta2 microglobulin was associated. We could separate out the classical H-2 antigens with further precipitations using monoclonal anti-class I antibodies of limited reactivity, and hopefully characterize whatever 40-45 kd proteins remained. This approach was unsuccessful since we failed to obtain cell suspensions of sufficient viability with intact surface proteins. These results should serve as a warning to others investigating cell surface proteins of cells obtained from tissues via enzyme digestion. In addition, Rossant and Croy (1985) have noted changes in the maternal/fetal distribution of different placental cell subpopulations obtained by mechanical disruption versus collagenase or trypsin digestion. The enzyme treatments apparently were selectively toxic to fetal cells. Cell surface H-2 antigens can be regenerated upon culture of the placental cells for 48 hours

(Athannasakis, *et al.*, 1987), but the number of H-2 positive cells in the initial populations is unknown. Thus it has not yet been determined whether the antigens are generally re-expressed, or that a small number of H-2 positive cells outgrow the rest of the population under the culture conditions.

Steady state mRNA levels can be a good indication of the expression of a given protein. mRNA can be extracted intact from cell suspensions or whole tissues in the presence of ribonuclease inhibitors. Since the entire population is quickly lysed after removal from the animal, there is no selection based upon the hardiness of different cell types with respect to isolation procedures, and thus a measure representative of the tissue can be obtained. We were able to purify high quality total cytoplasmic RNA from most gestational tissues with minimal degradation. The collection of molecular probes used to screen this RNA was unique in that the oligomeric DNAs could identify 29 of the 33 genes in the BALB MHC. Thus although attempts to identify class I molecules using biochemical techniques failed, we hoped to indicate antigen expression using the appropriate molecular probes.

The data show that all of the classical class I genes are expressed to some extent in mid to late gestational tissues (Table 8). H-2K^d was detected at approximately equal levels in all tissues throughout the period surveyed, but since the PrK cross reacts with a TI region gene, the ontogenic expression of this gene cannot be determined in this system. The H-2D probe (PrD) was strain specific and bound to B10 x BALB placental RNA from days 11 through 16. Only day 11 and day 12 yolk sac seemed to express this gene and the fetal liver was uniformly negative. Caution must be used in evaluating the data from fetal liver samples, since RNAse levels are highest in the liver, and these samples showed at least some degree of degradation. The L gene was just detectable in late (day 13-16) placenta and absent from yolk sac and liver. This is not surprising since H-2L antigen expression in the adult is low compared to H-2K or H-2D (Dower and Segal, 1985) and the H-2^b haplotype lacks an L gene altogether (Flavell, *et al.*, 1986).

Two of the other four genes located in the K/D region of the MHC were also apparently expressed. Pr2-2 identifies the D4 gene situated just centromeric to the L gene. This probe bound weakly to all tissues under low stringency only. Pr11-2, the probe specific for the K2 gene (which is centromeric to K), yielded the strongest signal on all tissues which persisted through a very high stringency (60^o C.) wash. This probe identified three

species of RNA, one of which co-migrated with the 18S band of ribosomal RNA (rRNA). Since Pr11-2 has some cross reactive binding to rRNA, part of this intense singnal was undoubtedly non-specific. The gene was found to be "expressible" in the DNA transfection studies of Goodenow and colleagues (1982), but has yet to be found in adult tissues (Lew, *et al.*, 1982; Flavell, *et al.*, 1986 and S. Hunt, personal communication). There was no evidence for the expression of D2 (identified by PrQ10), and there was no probe available for D3. It is interesting to note that these genes are more distal and opposite in orientation to the L gene than D4, which is expressed, although they may be close to D which is expressed at relatively high levels. Further studies on the effective distance of the L enhancer could provide explanations for this difference. Enhancers, unlike promoters, can act in either orientation over long stretches (> 10 kb) of DNA (Khoury and Gruss, 1983). However, some intervening sequences can interfere with the ability of the enhancer to cover long distances.

Probes existed for six of the eight genes in the Qa region. None of these probes hybridized to the RNA of gestational tissues. This was surprising since the Q10 gene is expressed in adult liver (Cosman, *et al.*, 1982). The ontogeny of Q10 has recently been studied (Stein, *et al.*, and Fahrner, *et al.*, 1987). Q10 message can be detected as early as day 12 in the liver and visceral yolk sac, with a peak of expression at day 14. Fahrner's group used a probe which identified the 5' region of the gene and Stein's group used a probe derived from the 3' untranslated region. The probe used in the work presented here was from the transmembrane region. Alternate splicing in the liver produces Q10 molecules with different cytoplasmic termini (Lalane, *et al.*, 1985) and a novelly spliced Q9 gene, which has deleted the fourth, fifth, sixth, seventh, and most of the eighth exon including the transmembrane region, has been isolated from a peri-implantation (day 8) cDNA library (Farhner, *et al.*, 1987). Thus, it is possible that Q10 is present in the RNA analysed here, but not detected by our probe. The 27.1 (Q7) gene is also apparently expressed in adult liver and spleen (Lalane, *et al.*, 1985). Qa-2 is found on bone marrow stem cells (Lynes, *et al.*, 1982) and thus would be a strong candidate for a prenatally expressed differentiation antigen. Yet the PrQ10 probe, which identifies the Qa-2 gene, did not hybridize to RNA from gestational tissues.

Four probes from the Tla region hybridized to the RNA blots. Of these, the PrT3/T13 probe identifies a serologically expressed gene, T13 which

encodes the TL antigen (Shen, *et al.*, 1982; Obata, *et al.*, 1985; Chen, *et al.*, 1985 and Pontarotti, *et al.*, 1986). The signal from this probe, however, was very heterogeneous as it did not identify a discrete size of message but bound to a smear of RNA, and did not survive the higher stringency wash. However, the T_m of the PrT3/13 probe is 54.3° C., and so the higher stringency wash may have been too close to the melting temperature to allow sufficient specific signal to remain hybridized to the filter. It is also worth noting that the binding of these probes to the cosmid clones from which they were derived was very clear cut, and that there was little or no cross hybridization to other genes except where specifically noted.

The binding of the PrT10/T18 probe was strong throughout mid and late gestation but also did not persist through the higher stringency wash (T_m of PrT10/18 = 58.2°). PrT1/T11 hybridized weakly to tissues from day 12 (but not day 11) onwards, and this binding persisted through the 50° C. wash. Since the antigens possibly encoded by these loci have not been identified, it is difficult to draw further conclusions from the expression of these RNAs. The binding of the PrT7/T15 probe was weak but persisted even through a very high stringency wash (60°). Tla gene expression is probably complex since the haplotypes have overlapping specificities (Old and Stockert, 1977 and Flaherty, 1981). The complexity of this region is also reflected by the fact that TL negative mouse strains can produce TL positive leukemias. The molecular genetic basis of this phenomenon is now understood. Although Tl$^-$ strains lack some of the genes encoding the Tl antigen, highly homologous genes are activated in the tumors and produce a class I molecule which reacts with the anti-TL monoclonal antibody (Obata, *et al.*, 1985; Chen, *et al.*, 1985 and Pontarotti, *et al.*, 1986).

The molecular genetics of the Qa/Tla region has produced a great amount of information about these genes. Investigators studying restriction fragment length polymorphisms (RFLPs) in the region confirmed its limited polymorphism (Steinmetz, *et al.*, 1981 and Margulies, *et al.*, 1982) and revealed evolutionary relationships amoung the Qa/Tla antigens. Sequence information has confirmed that gene duplications and subtractions frequently occur in the MHC (Lew, *et al.*, 1986; Flavell, *et al.*, 1986 and Klein and Figueora, 1986) , and there is ample evidence for unequal crossing over, gene conversion, and point mutation as mechanisms to generate diversity. Within the B6 MHC, Q5, Q7 and Q9 are similar, as well as Q6, Q8 and Q10, indicating duplication of gene pairs

(Weiss, *et al.*, 1984). In addition, the H-2K^b and H-2K1^b genes appear to be derived from the Q6, Q7 gene pair. There are also apparent duplications in the BALB Tla region (Fisher, *et al.*, 1985): T11, T12 and T13 are highly homologous to T1, T2 and T3, and the genes T6 through T9 are similar to T14 through T17. Indeed, many of the oligonucleotide probes used in the study reflect these homologies in their pattern of cross reactivity (see Results). The naturally occurring "bm" series of mutants producing differences in the H-2K^b gene are generally believed to have originated by gene conversion using several Qa region genes as donor sequences (Pease, *et al.*, 1983; Geliebter, *et al.*, 1986 and Mellor, 1986).

Evidence addressing the extent to which these related genes are essentially "interchangable" must wait for complete genetic and molecular analyses of the promoters, enhacers and other regulatory elements controlling transcription. Exon shuffling experiments have shown that the defects in Q6, Q7, Q8/9 and Q10 expression may be due to controlling elements located in the 3' portion of the genes (Straus, *et al.*, 1985 and Stroynowski, *et al.*, 1985). There is no consensus from the work presented here that argues strongly for differential activation of any given gene during mid to late gestation. However, the coordinate expression of the genes neighboring the classical H-2K and H-2L genes suggests powerful, long range, cis-acting activators. The detection of Tla specific RNA in the complete absence of Qa gene transcription is intriguing, especially considering that at least two Qa gene products, Qa-2 and Q10, have been identified in adult tissues.

Diversity can also occur post transcriptionally. Alternate splicing of heterogeneous nuclear RNA (hnRNA) can produce different messages from the same precursor. Brickell, *et al.*, (1983) obtained H-2D^d cDNA clones from SV-40 transformed fibroblasts that were full length or had deleted exon VII. Others (Lew, *et al.*, 1986 and McCluskey, *et al.*, 1987) isolated such variants from L-cells transfected with intact H-2K^b or H-2K^d genes. Thus, it appears that MHC molecules have the capacity for alternate cytoplasmic tails and/or transmembrane regions. These variants could give rise to differing responses (depending upon intracellular conditions) to the same external stimuli (e. g. binding to the N terminal of the molecule). The significance of these different forms has yet to be explored. It is worth noting that any such variation would possibly be overlooked in our study since the probes were specific to the

transmembrane of the MHC molecule, which could be deleted if polyadenylation sites 5' of the transmembrane exons are used in producing the relevent mRNA.

Conclusions

The "riddle of the fetal allograft" has not been solved. The uterus is not a privileged site: graft rejection can easily occur within the uterus, although the presence of decidua or gestational hormones can retard the process. The trophoblast itself seems capable of immunoregulation since ectopic pregnancy can proceed until late in gestation. The fetus and placenta are hypoantigenic for class I and class II histocompatibility antigens, but class I antigens have been identified in rodent spongiotrophoblast and human extra-villous trophoblast, tissues in intimate contact with maternal cells. In addition, non-traditional class I antigens may be expressed at high levels, and there are Ia positive cells (presumably capable of processing and presenting antigen) in the maternal tissue immediately surrounding the conceptus throughout most of gestation. The trophoblast has been shown to both bind and elicit anti-OFA and/or stage/tissue-specific antibodies. Fetal cells can enter the maternal circulation and even colonize maternal organs on a long term basis, thus providing a persistant antigenic challenge. Humoral and cellular immunity exists as a result of pregnancy, although the cellular responses are especially difficult to demonstrate in primigravida. Pregnancy serum proteins can be potent nonspecific inhibitors of immune responses, but only at high concentrations. Whether or not such effective concentrations exist at the maternal-fetal interface is debatable. Systemic suppression is not a common feature of pregnancy, but local, active, cell-mediated suppression seems an essential concomitant of successful pregnancy: some xenogeneic and allogeneic pregnancy failures are invariably associated with a lack of decidual suppressors and a large infiltrate of cytotoxic cells. The stimulus for the generation of these cytotoxic killers is not entirely clear; the killer cells could be responsible for fetal death, or, as has been suggested by Croy, Rossant and Clark (1982), be recruited to an area of dead tissue as a "mop-up" mechanism. Thus, the *in vivo* relevance of these suppressor phenomena remains to be demonstrated. The placenta can be a sink for specific antibody directed against the alloantigens of the conceptus, but may be of limited capacity. Cellular traffic across the normal, intact placenta is apparently restricted to red blood cells; maternal lymphocytes have only been convincingly identified in cases of graft-versus-host disease or other immunodeficiency in the offspring. This does not

mean that the placenta is absolutely impermeable to sensitized cells; our best detection techniques can resolve only 1 in 10^5 cells. Circulating anti-paternal antibody that can block the generation of the more destructive cell-mediated response is found in about fifty percent of human pregnancies. Such antibody is absent from mice, horses and humans undergoing spontaneous abortion of unknown etiology, but these immunocompromised females can be successfully treated by generating the blocking antibody with lymphocyte immunizations, or passive transfer of immune serum. Females immune to paternal cells often produce slightly larger placentae (but smaller decidual swellings) than naive mothers or those tolerant of paternal antigens. T-cell derived growth factors enhance the *in vitro* growth of placental cells, and depletion of mature T-cells from a pregnant female can compromise pregnancy. Yet, mice congenitally deficient in T-cell responses can reproduce, so we are not dealing with an all or none phenomenon.

It is becoming clear that a functional immune system is active within the pregnant female, yet what determines the balance between immunological rejection and suppression is not yet known. Indeed, we are not even clear about the initiation of such a regulatory pathway, the immunogenic stimulus. The work summarized in this thesis attempted to elucidate the identity of that stimulus. Firstly, we demonstrated that the placenta excludes potentially alloreactive cells from access to the fetus. While RBC can cross the placenta in low numbers, only around 200 maternal splenocytes could enter the fetal liver via transplacental passage. The only exceptions to this rule were two cases in which very large maternal infiltrates into the fetus were observed. Since the animals were sacrificed in order to do the assay, we could not monitor these animals for the onset of graft versus host disease.

The nature of the paternal antigens expressed was investigated. Preimplantation blastocysts may express some form of non-traditional class I antigen; mature placentas absorb anti-class 1 monoclonal antibodies from maternal circulation although it did not appear that non-tradiontional (*Qa/Tla*) determinants were present.

Analysis of the RNA isolated from gestational tissues showed that all of the classical MHC antigens (H-2K, D, and L) were expressed in mid to late gestation. In addition, the neighboring genes in the K and D regions were expressed, but two genes slightly more distal to D and in the opposite orientation did not show evidence of expression. Klein's (1983) suggestion that

this represents a "loose" control over the essential expression of the traditional class I antigens is therefore intriguing. Several genes in the *Tla* region were expressed, although none of these genes varied in the ontogeny of their expression with the possible exception of T1 (or T11) which was not detectable on day 11 placenta but was expressed from day 12 onwards. Only one serologically detectable protein has so far been described as deriving from this region (the TI determinant is encoded by T3 or T13), and that gene is one of those identified here in gestational tissue. No *Qa* region transcripts were detected even though at least two of these genes (D4 and Q10) produce proteins that are commonly found in adult tissues. If only a small percentage of gestational cells express these genes, however, the transcripts would be too dilute to produce a signal with the radioactive probes at the sensitivity of our assay. The definitive experiment to perform would be *in situ* hybridization of cDNA probes to tissue sections from mouse embryos. This type of study could reveal expression on small localized areas that might be missed in assaying the entire tissue as a pool.

The traditional class I antigens (K, D and L) remain the most likely candidates for stimulation of the maternal anti-fetal response. Some genes neighboring these genes may also be important antigens, as well as a few TI region genes. These determinants probably exist in sufficient amounts to trigger immune reactivity. It seems clear that future studies should be directed toward examining the regulation of this response, especially since the non-traditional antigens preferentially ellicit a non-destructive type of antibody isotype while the traditonal determinants generate compliment-binding IgG_2.

It is clear that we have accumulated much information but found no single reason for the survival of the semiallogeneic conceptus. Self-recognition and immune responses preceeded viviparity by many evolutionary ages, and it is probable that the mammals employed many different mechanisms in parallel in order to avoid rejection of the feto-placental unit. Indeed, the immunological balance in any particular species may be as varied as its distinctive placental ontogeny and ultrastructure. In addition, immune recognition, activation, clonal expansion and effector function have fairly reproducible kinetics across species lines, but mammalian gestation varies from 19 days in the mouse, to two years in the elephant. Gill (1983) has pointed out that the difficulty in obtaining and maintaining inbred strains may indicate that homozygous stocks currently available represent a minimum requirement for successful gestation. We now

need to determine whether the immunological mechanisms operating during gestation are aimed at suppression of a response, or the redirection of the immune response into the elliciation of the growth promoting lymphokines elaborated by sensitized T-cells. There is evidence that both mechanisms are operating, and indeed, they are not mutually exclusive. The studies summarized in this thesis have shown that class I antigens, the primary immunogens in many transplant rejection reactions, are present at the maternal-fetal interface. The precise cellular location of these antigens, and the relative importance of the traditional versus the non-traditional determinants await future investigations.

Table 1. Monoclonal Antibodies Recognizing Class I MHC Determinants

hybridoma	immunization	specificity (cross rxs.)	antibody subclass	ascites titer (pos. strain)	ascites titer (cross rx. strain)	reference
11-4.1	BALB α CKB	H-2K^k (H-2 p, q, r)	IgG2a, k	>(2 x 10)$^{-12}$ (C3H)	--	Oi, *et al.*, 1978
16-3-1N	C3H.SW α C3H	H-2K^k (H-2p, q, r)	IgG2a, k	(2 x 10)$^{-11}$ (C3H)	--	Ozato, Mayer, and Sachs, 1980
B8-24-3	BALB α EL4	H-2K^b (H-2p)	IgG$_1$, k	(2 x 10)$^{-12}$ (B10)	--	Kohler, *et al.*, 1981
20-8-4S	C3H α C3H.SW	H-2K^b/D^b (H-2K$^{d, r, s}$)	IgG2a, k	(2 x 10)$^{-17}$ (B10, BALB)	--	Ozato and Sachs, 1981
28-8-6S	C3H α C3H.SW	H-2K^b/D^b	IgG2a, k	(2 x 10)$^{-12}$ (B10)	--	Ozato and Sachs, 1981
34-5-8S	C3H α BD/F$_1$	H-2D^d (H-2L^d)	IgG2a, k	>(2 x 10)$^{-12}$ (BALB)	(2 x 10)$^{-4}$ (B10)	Ozato, Mayer and Sachs, 1982
S19/8	SJL α, B10.S	β2μ	IgG2a, k	not done	not done	Tada, *et al.*, 1980
23-1.16	SJL α B10.S	β2μ	IgG2a, k	>(2 x 19)$^{-9}$ (B10)	(2 x 10)$^{-5}$ (C3H)	Chorney, *et al.*, 1982

Table 2. Summary of oligonucletide probe composition and specificity

probe	nucleotide sequence	specific binding
Pr11-2	CCGACTCCAACATGGTGACCA	$K2^d$, rRNA
PrK	TGTCTCCAACACGGTAATCA	K^d, "37"
PrD	TCCACCAAGACTAACACAGTA	D^d
PrQ10	TCCACCAAGACTAACAC	D2d (Qa2)
Pr2-2	ACACCAACATGGCCATCA	$D4^d$
PrL	CCGTCCACTGACTCTTAC	L^d, L^b
PrQ1	GGTCATCATTGGAGTTA	$Q1^d$
PrQ5	GGCGAACGTAGCTATTCT	$Q4^d$, $Q6^d$
PrQ6	GGCGACCATTGCTGTTGTT	$Q7^d$ (27.1)
PrQ7	GGCGACCATTGCTATTGT	$Q8/9^d$
PrQ8	GTCACACATTGCTGATCTGG	$Q10^{b,d}$
PrT1	GCCCACCAGGACCATTTG	$T1^d$, $T11^d$
PrT2	AGCCCATCATCTTCATT	$T2^d$, $T12^d$
PrT3	CCCAACAGGACCACTG	$T3^d$, $T13^d$
PrT4	GGACTCCAACATGGTAATCG	$T4^d$, $T6^d$, $T9^d$, $T14^d$, $T16^d$
PrT5	GTCCATCATCCTCATTAG	$T5^d$
PrT6	AATCATGGCTGTTCTTTT	$T6^d$
PrT7	ACACAGGTCGAACTTTGC	$T7^d$, $T15^d$
PrT9		$T9^d$
PrT10	CCTAGGACAGGTCGCCAT	$T10^d$, $T18^d$
PrT12	CCTCCTTAGCCCATCATCT	$T2^d$, $T12^d$
PrT17		$T17^d$, $T9^d$

genes without probes: $D3^d$, $Q2^d$, $Q5^d$, $T8^d$

Table 3. GPI analysis of Maternal to Fetal Trafficking

strain combination	days gestation 15	16	17	18	19	+1[1]
CC3/F_1 x BALB	0/5[2]	0/10	0/10	1/26[3]	0/15	0/30
B6A/F_1 x A/J	not done	0/10	0/14	0/16	1/22[3]	0/14

[1] One day postpartum.

[2] Ratios indicate the number of offspring in which trafficking was detected over the number of samples analyzed.

[3] One animal in this group showed a significant amount of trafficking.

Table 4. FACS Analysis of Maternal to Fetal Trafficking

	cells injected[1]	population sampled	# animals sampled	total # cells counted[2]	% faintly labeled[3]	% intensely labeled
1	–	mother's blood	1	120,880	0	0
2	–	mother's spleen	1	100,112	0.00099	0
3	–	neonate's blood	10	1,271,329	0.0022 ±0.0028	0.0012 ±0.0013
4	–	neonate's liver	10	909,499	0.094 ±0.047	0.0054 ±0.0053
5	10^9 lysed RBC	mother's blood	1	126,172	0.20	0
6	10^9 lysed RBC	mother's spleen	1	127,954	0.27	0
7	10^9 lysed RBC	neonate's blood	7	804,343	0.0029 ±0.0030	0.0015 ±0.00087
8	10^9 RBC	mother's blood	3	234,092	3.4 ±1.9	0.81 ±1.1
9	10^9 RBC	mother's spleen	1	80,612	7.4	0.03
10	10^9 RBC	neonate's blood	30	2,510,404	0.13 ±0.143	0.047 ±0.17
11	10^9 RBC[4]	neonate's blood	1	79,547	1.4	0.22
12	10^9 RBC[4]	neonate's blood	1	75,815	1.6	0.92
13	10^9 RBC[4]	neonate's blood	1	81,648	0.51	0.15
14	10^8 lysed WSC	mother's blood	1	84,551	0	0.0012
15	10^8 lysed WSC	mother's spleen	1	118,839	0.0059	0.00084
16	10^8 lysed WSC	neonate's liver	9	769,630	0.15 ±0.12	0.016 ±0.017
17	10^8 WSC	mother's blood	4	452,353	0.071 ±0.12	0.51 ±0.85
18	10^8 WSC	mother's spleen	4	422,636	0.0040 ±0.0039	0.0039 ±0.0056
19	10^8 WSC	neonate's liver	24	2,043,235	0.16 ±0.29	0.0086 ±0.013

[1] Controls included an uninjected control (lines 1 through 4), and a negative control (lines 5 through 7 and lines 14 through 16): 10^9 RBC or 10^8 WSC lysed by freeze-thaw, and injected intravenously into pregnant females. This provides a measure of the amount of free label binding *in vivo* to constitutive cells on the maternal or fetal side of the placenta.

[2] Total number of cells sorted by the FACS for all samples in each group.

[3] Since cells labeled to different degrees, the labeled cells were sorted into two populations: faintly or intensely labeled.

[4] Individual mice that did not show the sorting pattern representative of the group were also scored individually.

Table ... Indirect Immunofluorescence of Anti-class I Sera/Monoclonal Antibody on Pre- and Postimplantation embryos

antibody	Binding to: B10		BALB			C3H		
	oocyte	blastocyst	oocyte	blastocyst	implant	oocyte	blastocyst	implant
none	--	--	--	±	--	--	±	--
α H-2^b	--	ND[1]	ND	ND	ND	--	ND	ND
α H-2^d	--	--	--	+	-/+[2]	ND	±	-/+[2]
α H-2^k	--	ND	ND	ND	ND	--	+	--
11-4.1	--	--	--	--	ND	--	--	ND
16-3	ND	ND	--	ND	ND	--	--	--
28-8	--	--	ND	ND	ND	ND	ND	ND
34-5	ND	ND	--	--	--	--	ND	ND
S19/8	--	+	--	--	ND	--	ND	ND
23-1.16	--	--	--	ND	ND	ND	--	ND

1 Not done.
2 Fibroblast-like (trophoblast) cells did not stain. The layer of round cells delaminating from the ICM stained brightly.

Table 6. Binding of ^{125}I-anti class I antibodies to gestational tissues.

mating combination	Mab. injected	cpm injected	# fetuses	cpm/g tissue DC	YS	placenta	fetus
C3H x C3H	20-8-4S (75%)	4×10^5	8	5900	31,000	19,000	3000
"	"	"	7	8300	24,300	14,400	3100
"	"	"	6	9400	35,900	19,000	3600
"	"	"	7	8000	37,700	22,500	3300
"	"	"	8	5300	20,200	10,500	1800
"	"	"	8	5900	26,800	13,700	2600
"	"	"	3	11,200	30,500	19,300	1700
mean			7	7700	29,500	16,900	2700
(±stnd. dev.)			(±2)	(±2200)	(±6200)	(±4100)	(±700)
C3H x B10	20-8-4S (75%)	4×10^5	3	19,300	22,800	41,100	1500
"	"	"	5	23,400	27,800	69,400	1500
"	"	"	7	4700	9400	18,600	700
mean			5	15,800	20,000	43,000	1200
(±stnd. dev.)			(±2)	(±9800)	(±9500)	(±25,500)	(±500)
C3H x C3H	28-8-6S (27%)	7×10^5	10	6600	30,500	12,200	2400
"	"	"	6	7400	41,700	12,300	2300
"	28-8-6S (46%)	5×10^5	7	5600	32,400	14,100	2100
"	"	"	9	5600	36,400	13,600	1900
"	"	"	6	6300	27,000	11,500	2100
"	"	"	5	6200	30,000	13,700	2500
"	"	"	7	7700	34,000	11,400	2300
mean			7	6500	33,100	12,700	2200
(±stnd. dev.)			(±2)	(±800)	(±4800)	(±1100)	(±200)
C3H x B10	28-8-6S (27%)	7×10^5	6	14,000	31,500	26,000	2700
"	28-8-6S (46%)	5×10^5	5	10,300	40,900	31,900	2600
"	"	"	5	13,800	53,400	40,300	4000
mean			5	12,700	41,900	32,700	3100
(±stnd. dev.)			(±1)	(±2100)	(±11,000)	(±7200)	(±800)

Table 7. Class I oligonucleotide probes react with total cellular RNA from gestational tissues.

strong binding	weak but persistant binding	transient binding	genes not expressed	genes without probes
K	L	D4	D2	D3
K2(rRNA)				
D				
			Q1	Q2
			Q4	Q5
			Q6	
			Q7	
			Q8/9	
			Q10	
	T1 or T11	T3 or T13	T2	T8
	T7 or T15	T10 or T18	T4	
			T5	
			T6	
			T9	
			T12	
			T14	
			T16	
			T17	

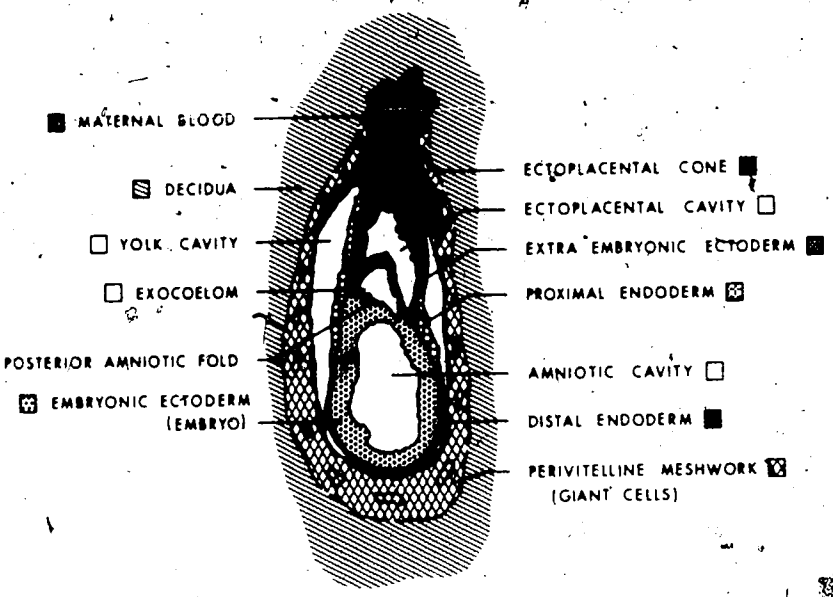

FIG 1 MOUSE EMBRYO, 7.5 DAYS POST-CONCEPTION

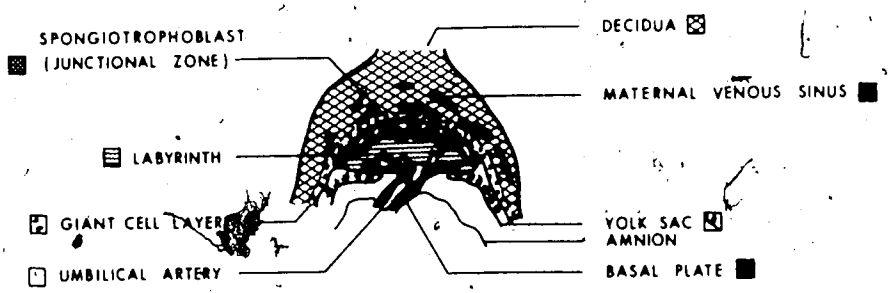

FIG 2 MOUSE EXTRAEMBRYONIC MEMBRANES, PLACENTA, AND DECIDUA, 12.5 DAYS POST-CONCEPTION

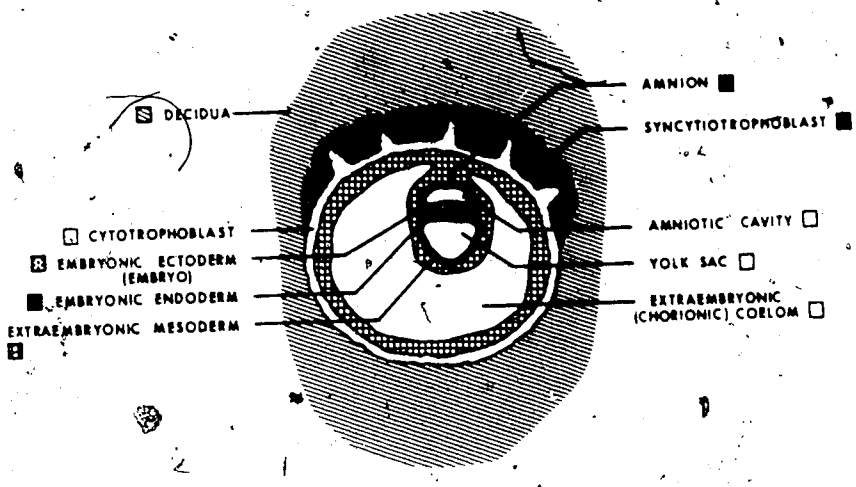

Figure 3. Peri-implantation human embryo, 15 days postconception.

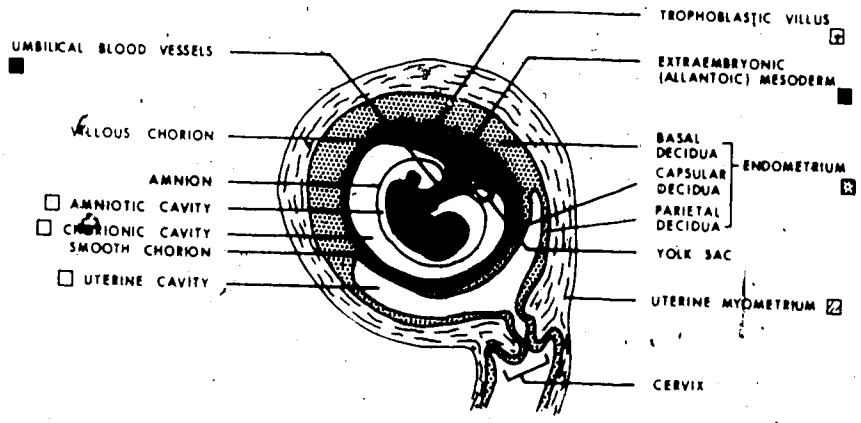

Figure 4. Late first trimester human conceptus, 8 weeks postconception.

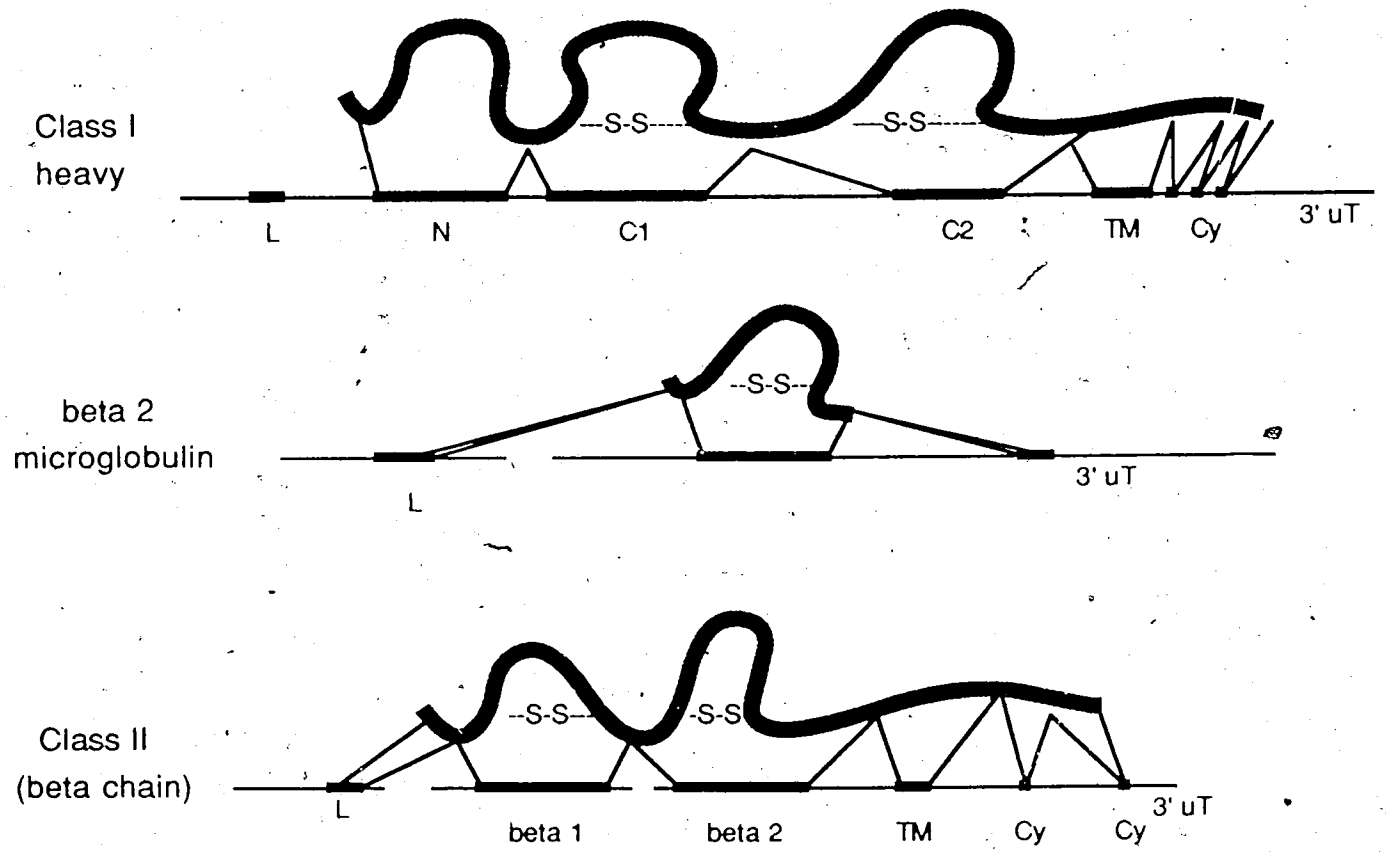

Figure 5. Exon-domain correspondence of class I and class II genes. Thick lines represent coding blocks (exons) seperated by thin lines of intervening sequences (introns). Line breaks represent large areas of DNA not shown. --S-S-- denotes intra-domain disulfide bonding. Note that the leader (L) exon of beta 2 microglobulin and the class II beta chain encode small protions of the amino terminal domain in the mature protein. The class II alpha chain organization is similar to that of the beat chain shown here, although the alpha 1 domain lacks a disulfide bridge.

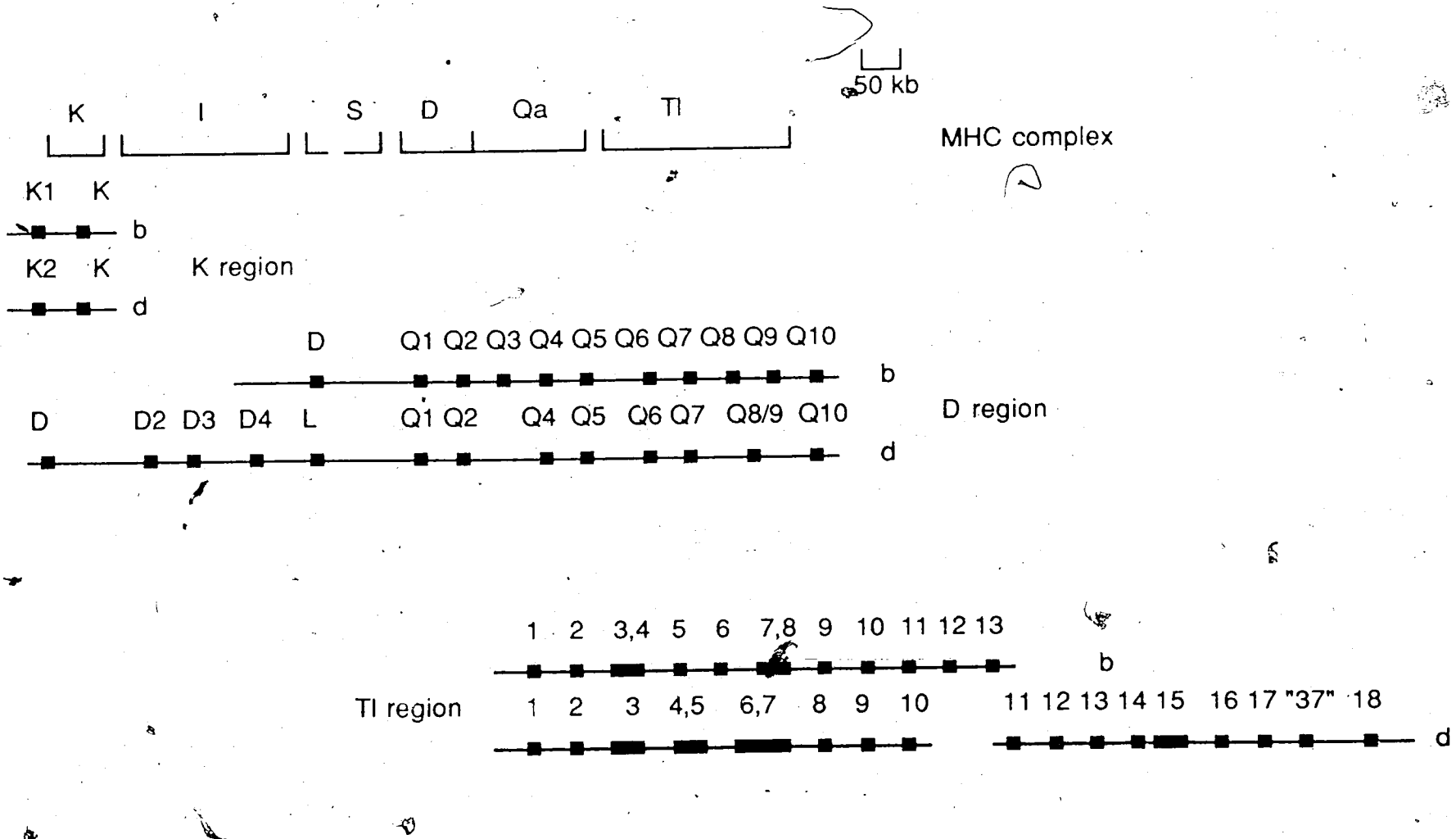

Figure 6. Schematic molecular map of the MHC in the B6 and BALB/c strains.
Boxes indicate genes/pseudogenes, lines indicate spacer DNA between coding blocks.

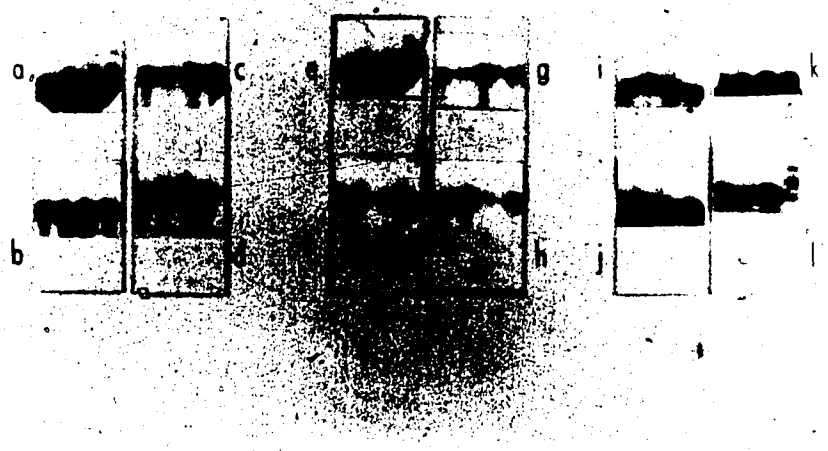

Figure 7. Electrophoretic analysis of GPI isozyme patterns. Frames a-d: fetal/neonatal blood, frames e-h: fetal/neonatal liver, frames i-l: fetal/neonatal spleen. Artificial mixture standards (frames a, e, and i from left to right): lane 1: 0% *Gpi-1 a/b*, lane 2: 1% *Gpi-1 a/b* lane 3: 2.5% *Gpi-1 a/b*, lane 4: 5% *Gpi-1 a/b*. Experimental samples frames b, c, d, f, g, h, j, k, and l. Note the characteristic pattern in lane 4 of frame l.

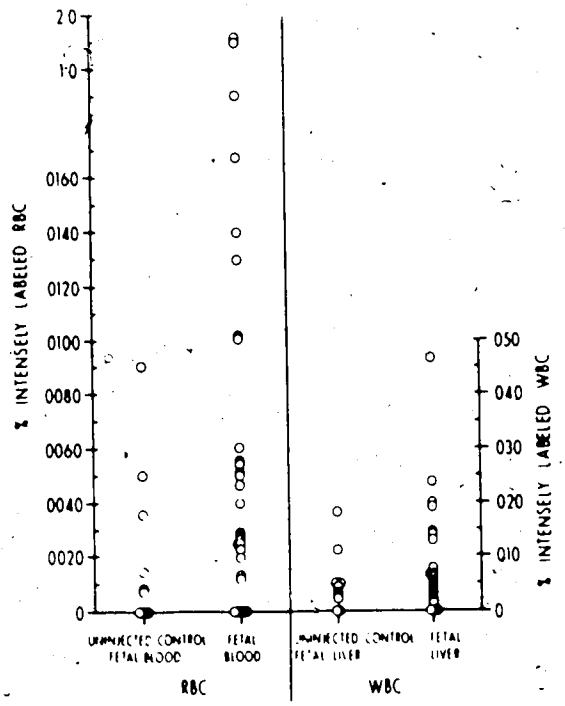

Figure 8. Distribution of FITC-labeled cells detected in fetal tissues. Left panels: scatter of samples from peripheral blood or the liver mononuclear cell fraction of ten neonates (from a single litter) individually sorted to provide a measure of machine background. RBC uninjected control, mean = 0.0012 ± 0.0013, WSC uninjected control, mean = 0.0054 ± 0.0053. Right panels: scatter of experimental samples. Thirty neonates (from three letters) screened for the presence of labeled RBC, and twenty-four neonates (from four litters) screened for the presence of WSC.

Figure 9. Binding of H-2 oligonucleotide probes to total RNA from gestational tissue.

Plates 1-4: All samples were electrophoresed on four different gels in lanes #1-8 (left to right) as follows: d13-16 plac blot: lane 1, day 13 placenta, B10 x BALB; lane 2, day 13 placenta, B10 x B10; lane 3, day 14 placenta, B10 x BALB; lane 4, day 14 placenta, B10 x B10; lane 5, day 15 placenta, B10 x BALB; lane 6, day 15 placenta, B10 x B10; lane 7, day 16 placenta, B10 x BALB; lane 8, day 16 placenta, B10 x B10. d13-16 liver blot: arranged as above for liver samples. d13-16 YS blot: arranged as above for yolk sac samples. d11/12 plac/YS: lane 1, day 11 placenta, B10 x BALB; lane 2, day 11, B10 x B10, lane 3, day 12 placenta, B10 x BALB; lane 4, day 12 placenta, B10 x B10; lane 5, day 11 yolk sac, B10 x BALB; lane 6 day 11 yolk sac, B10 x B10; lane 7, day 12 yolk sac, B10 x BALB, lane 8, day 12 yolk sac, B10 X B10.

Plate 1: PrK, PrQ1, PrT4; 37° wash.

Plate 2: PrQ7, PrQ10, PrT3/13; 37° wash.

Plate 3: PrT9, PrT10, PrT12, PrT17; 37° wash.

Plate 4: PrL, PrT1, PrQ5, PrT3/13; 50° wash.

Plate 5: lanes are indicated on the plate. 50° wash.

Plate 6: lanes arranged as for plates #1-4. PrD, PrQ6, PrQ8; 37° wash.

Plate 7: lanes arranged as for plates #1-4. Pr2-2, PrT2, PrT5; 37° wash.

Plate 8: lanes arranged as for plates #1-4 for PrT7. Pr11-2 and PrT6: d10, 11, 14, 16 plac blot: lane 1, day 10 placenta, B10 x BALB; lane 2, blank; lane 3, day 11 placenta, B10 x BALB; lane 4, day 11 placenta, B10 x B10 lane 5, day 14 placenta, B10 x BALB; lane 6, day 14 placenta, B10 x B10; lane 7, day 16 placenta, B10 x BALB; lane 8, day 16 placenta, B10 x B10. d12, 13, 15 plac blot: lane 1, blank; lane 2, day 12 placenta, B10 x BALB; lane 3, day 12 placenta, B10 x B10; lane 4, day 13 placenta, B10 x BALB; lane 5, day 13 placenta, B10 x B10; lane 6, day 15 placenta, B10 x BALB; lane 7, day 15 placenta, B10 x B10; lane 8, blank. d10, 11, 14, 16 YS: arranged as for d10, 11, 14, 16 plac blot, except, lane 1, blank; lane 2, day 10 yolk sac, B10 x B10. d12, 13, 15 YS: arranged as for d12, 13, 15 plac blot, except, lane 2, blank. d10, 14, 16 liver blot: arranged as for d 10, 14, 16 plac blot, except, lane 2, day 10 liver, B10 x B10; lanes 6 and 7, degraded. d12, 13, 15 liver blot: arranged as for d12, 14, 15 plac blot, except, lane 3, degraded; lane 7, blank.

Plate 9: same hybridization as plate #8; 50° wash.

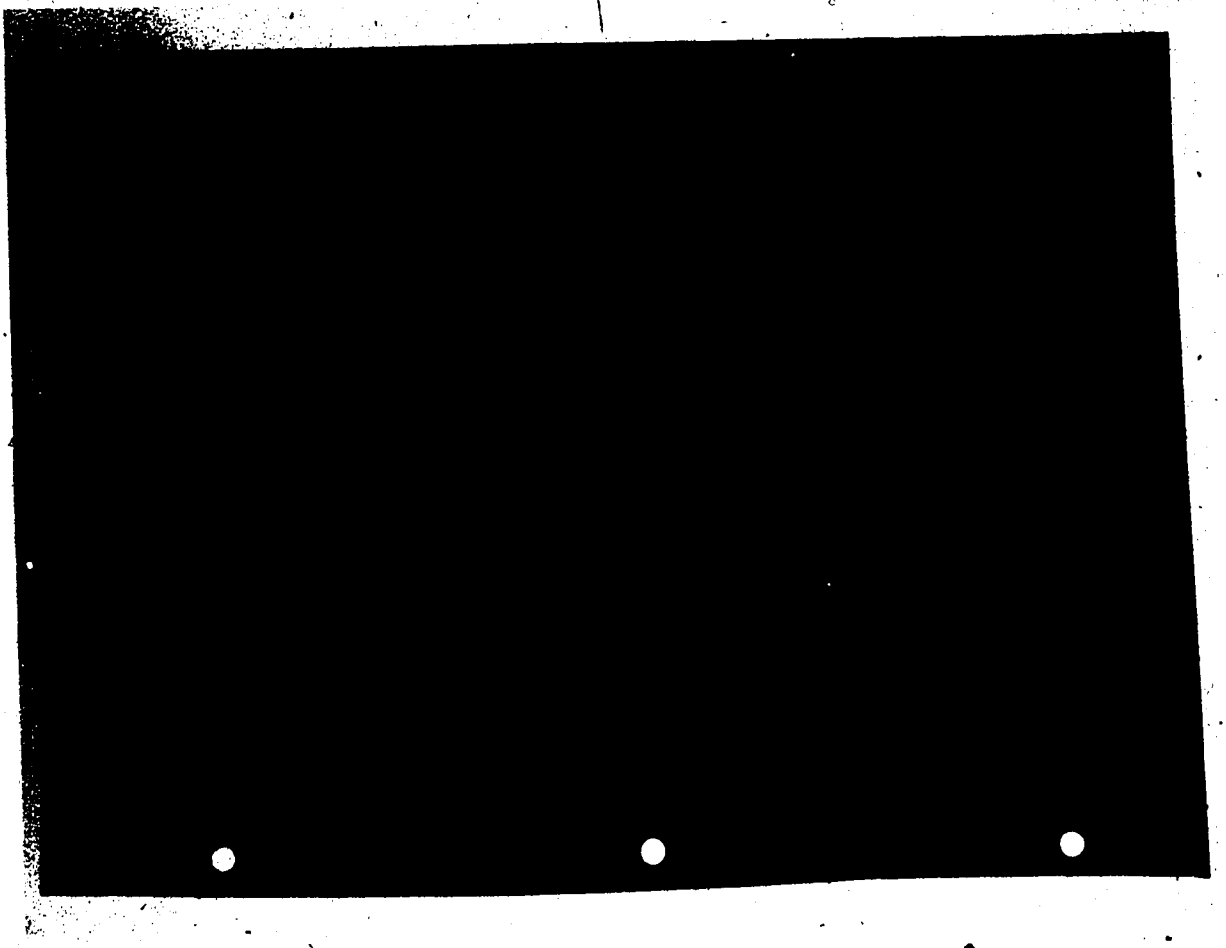

Figure 9, plate 1.

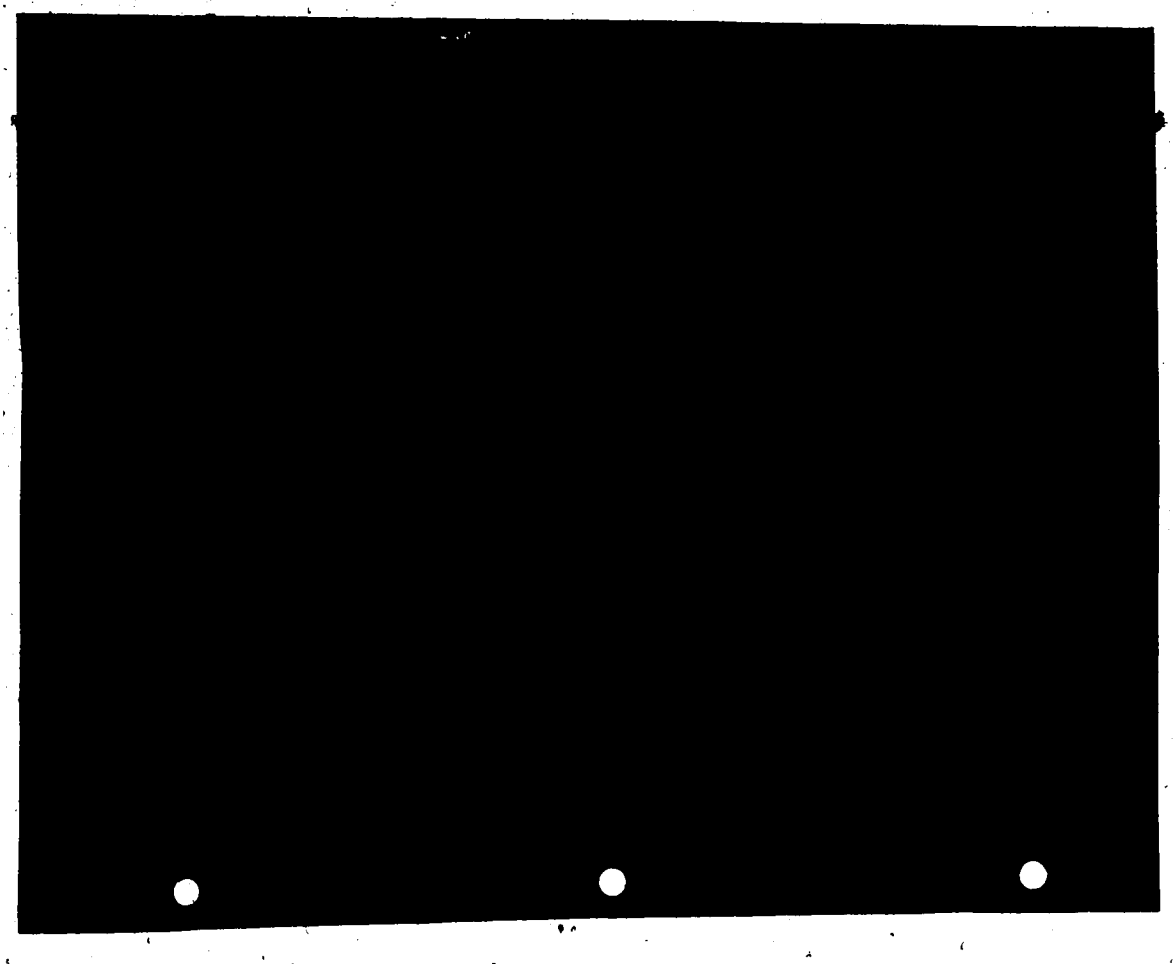

Figure 9, plate 2.

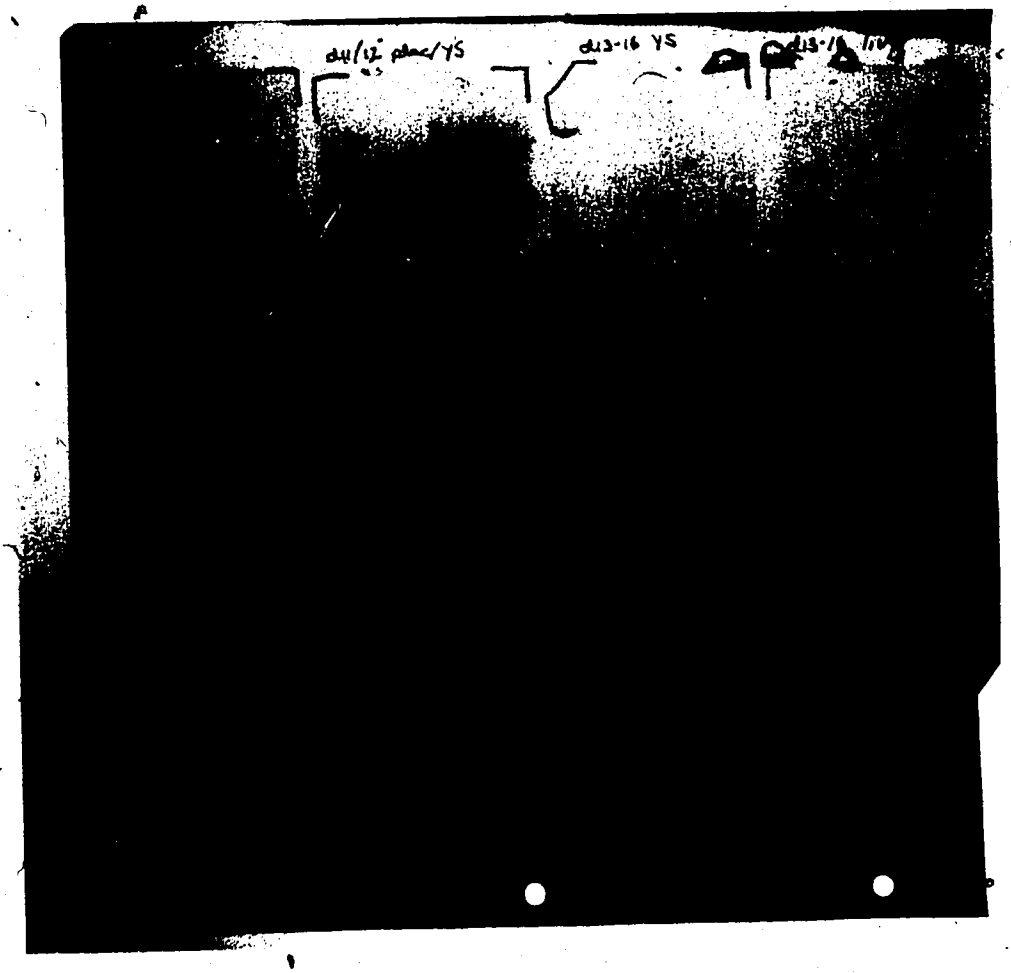

Figure 9, plate 3.

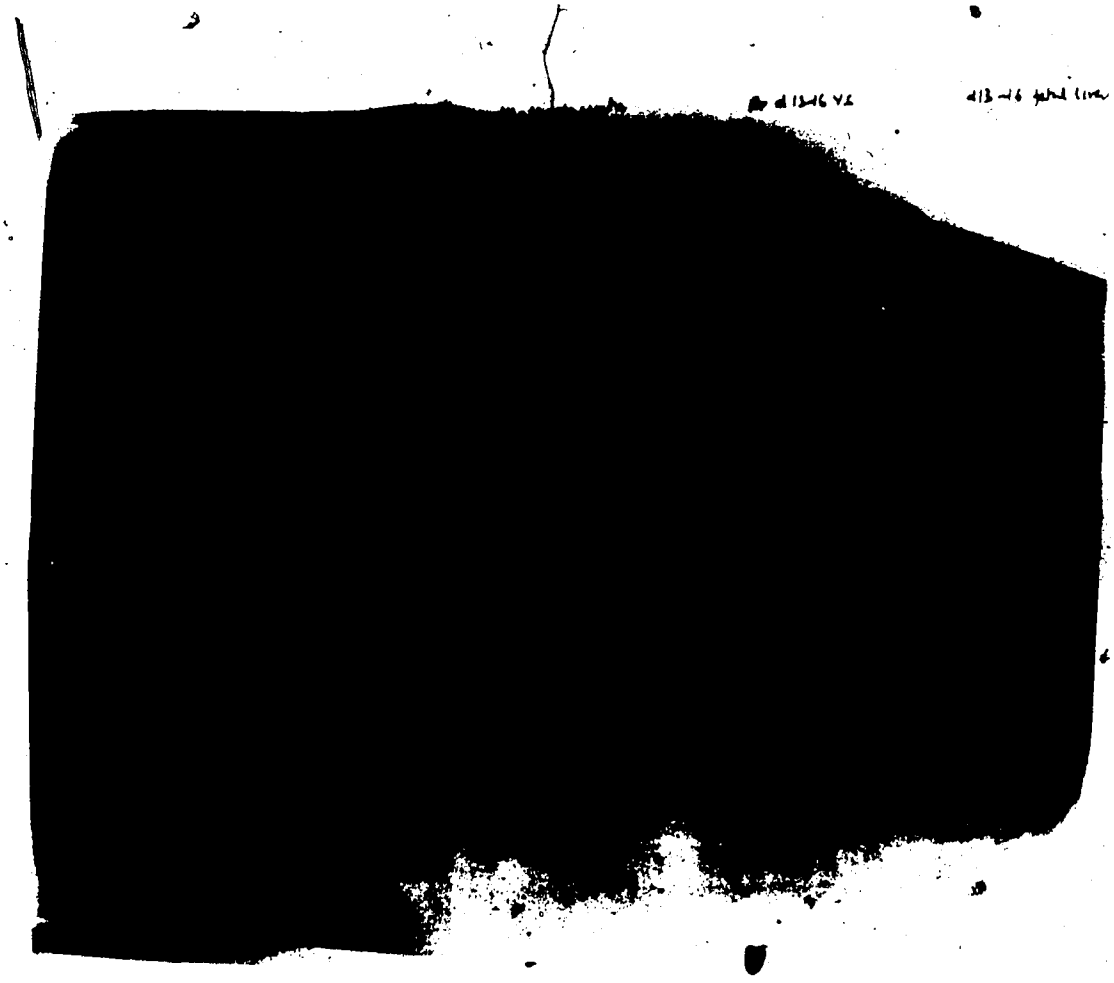

Figure 9, plate 4.

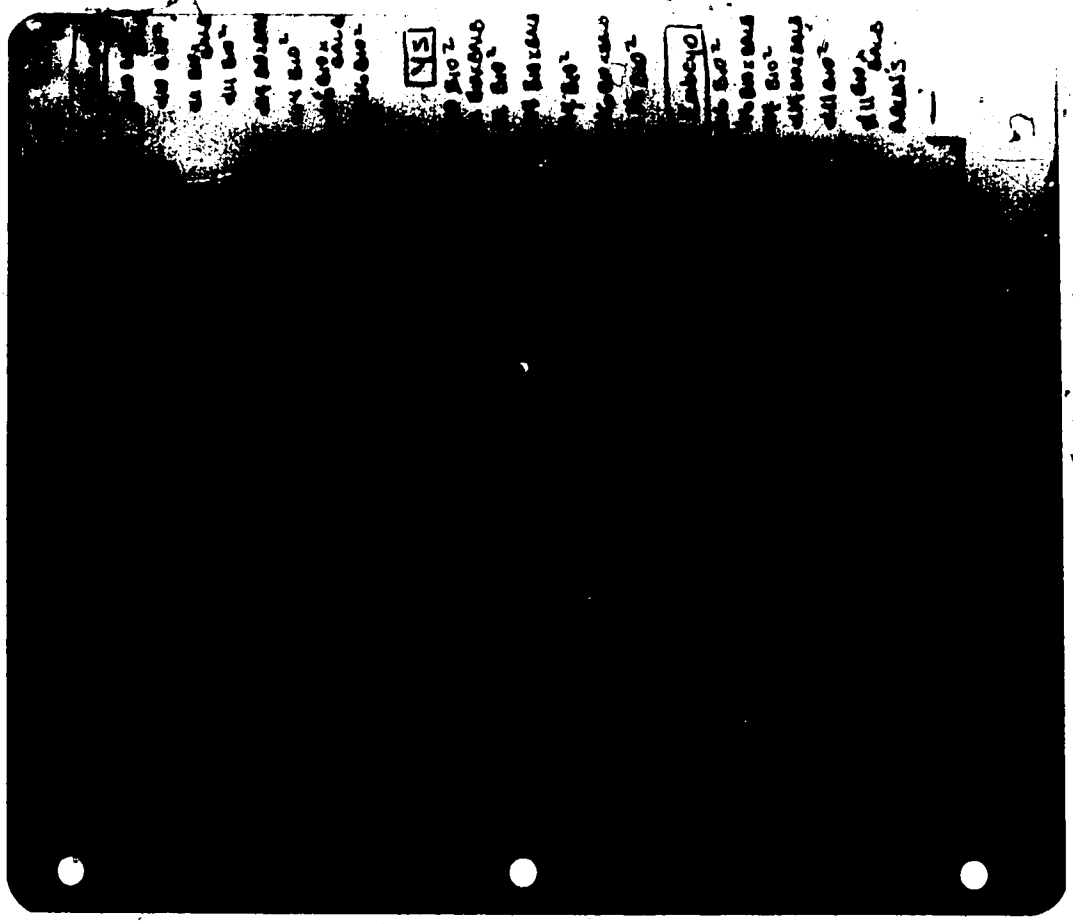

Figure 9, plate 5.

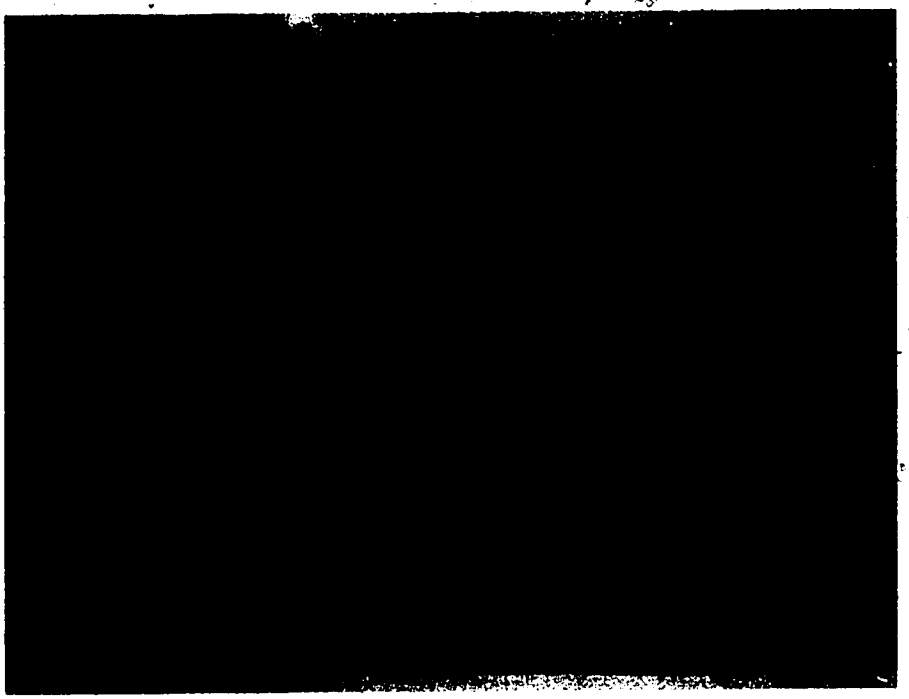

Figure 9, plate 6.

Figure 9, plate 7.

Figure 9, plate 8.

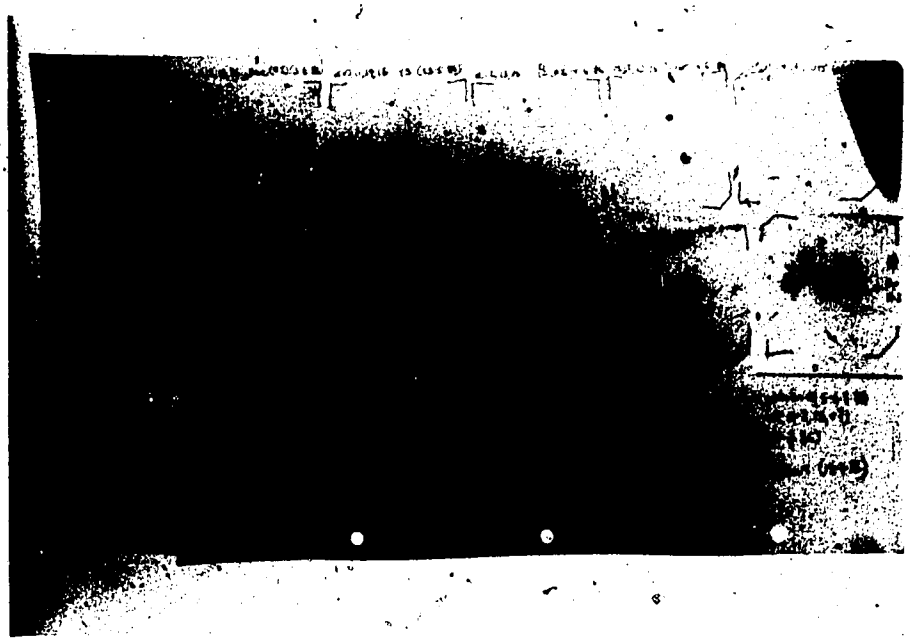

Figure 9, plate 9.

B L A N K P A G E

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