STUDIES ON THE IMMUNOLOGICAL INTERACTION BETWEEN MOTHER AND FOETUS

by

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Abstract

Studies have been made of the immunological interactions that occur between inbred female mice and their hybrid foetuses.

The effectsof maternal sensitization to paternal antigens have been examined. Contrary to earlier reports, it appears that allogeneic immunization has no effect on placental size at the eighteenth day of pregnancy but causes a small consistent depression of foetal weight. Reduction of litter size, and implantation number, and an increase in foetal death are sometimes but not always apparent. The immunization of the mother to Peromyscus antigens also reduces litter size and increases the number of foetal deaths. This suggests that non-specific factors of immunization may be responsible. Non-specific immunization also depresses foetal weight but the effect is smaller than that caused by specific immunity. It is suggested that further experiments involving immunization should take into account the influence of non-specific factors.

Although the mechanisms protecting the foetus from maternal sensitization are efficient in the later stages of pregnancy, the early stages appear to be more susceptible to interference.

Injections of the enzyme hyaluronidase, which increases placental permeability, have no effect on hybrid foetal and placental weight at the eighteenth day, but tend to increase the numbers of early foetal deaths. The effect is restricted to mother's sensitized to paternal antigens.

The removal of the spleen from allogeneically immunized females also fails to affect foetal and placental weights, and increases early foetal mortality. On the basis of these, and other observations, it is proposed that the presence of "enhancing" antibodies may play a role in protecting the early foetus.

A study was made of the effects of active maternal sensitization, and passively transferred antiserum, on the development of the uterine decidual cell response at the seventh day of pregnancy. The results show that active immunization to paternal antigens significantly reduces the size of the decidual response. The effectiveness of passively transferred antisera demonstrates that humoral, rather than cellular, components are responsible. The use of different methods of active immunization gives evidence that the diminished response is dependent on a particular kind of antibody. The strength of antibody in individual maternal serum determines the degree of decidual inhibition.

Inbred and hybrid eggs were cultured in the presence and absence of antiserum. The results show that whereas transplantation antigens can be demonstrated on the surface of oviducal embryos, hybrid embryos appear to be deficient in the expression of paternal antigens.

The problem of the foetus as a homograft is discussed in the light of present knowledge.

Contents

Chapter I

I n t r o d u c t i o n

Introduction

In most vertebrates the experimental transplantation of living tissue from one individual to another of the same species is normally unsuccessful. The widespread existence of genetic polymorphism for transplantation antigens ensures that in the majority of instances antigenic differences exist between any two organisms. Normally the presence of foreign tissue initiates an immune reaction that almost invariably leads to its destruction. This law of immunology is violated by one natural homograft - the foetus. The foetus possesses antigens received from its father which are foreign to the mother. The mother possesses antigens which the foetus has not received. As knowledge has been gained about the fate of normal tissue grafts, biologists have become increasingly intrigued with the success of the foetus as a homograft. In 1964 Billingham said "The apparently unqualified success of the foetus as a homograft has been recognized as one of the major unsolved problems of transplantation biology for more than forty years."

1. The foetus as a homograft

a) The antigenicity of the foetus

The antigenic nature of the embryo, except in the very early stages of development, has been well established. Cells from the liver of a mouse embryo thirteen days old will induce antibody production when injected into an adult mouse (Moller, 1963a) and embryonic tissues from a nine day-old mouse, transplanted into a pre-immunized allogeneic host, will undergo a classic homograft rejection (Edidin, 1964). Whether embryonic antigens are similar to adult transplantation antigens or are specifically foetal or tissue

specific has been disputed. The cytotoxic effect on the foetus of anti-sera resulting from hyper-immunization with malignant tissue (Möller, 1961; Schlesinger, 1965) does not prove the importance of transplantation antigens, because embryonic and neoplastic tissues may have other antigenic determinants in common (Abelev, 1963; Gold and Freedman, 1965; Prehn, 1967). In studies specifically involving H-2 antisera produced against normal tissue Pizarro et al. (1961) failed to demonstrate these antigens in newborn tissues, but Schlesinger (1964), using a different technique, did describe the absorption of H-2 agglutinins by ten and a half day foetuses.

There is some evidence to suggest that embryonic tissues might express embryo-specific and transient developmental (phase-specific) antigenicity. Experiments in several mammals have indicated that embryonic tissues possess antigenic factors which are characteristic of a particular period of morphogenesis and are absent from the adult animal (see review by Volkova and Maisky, 1969).

In general embryonic tissues appear to be immunogenically less reactive than those of adults. In mice, skin homografts from infant donors survive longer than similar adult grafts on H-2 incompatible recipients (Billingham and Silvers, 1964).

After the formation of the placenta the antigenicity of this organ, and in particular the foetal component of trophoblast, is of greater relevance to the success of the foetus as a homograft than the tissue of the conceptus. It has been established by grafting allogeneic placental tissue to specifically preimmunized recipients that the placenta is susceptible to rejection (Simmons and Russell,

1962; Uhr and Anderson, 1962). Hašková (1963) however, claimed that F_1 hybrid placental tissue was deficient in paternal transplantation antigens. Most of the experiments involving placental grafts have been criticized because the grafts are likely to have contained contaminating material.

The trophoblast is the only component of the conceptus to come into direct contact with maternal tissue. Several theories have been advanced to account for the apparent lack of antigenicity found in this tissue. They will be considered in a later section (see p. 8).

The antigenicity of the early embryo has been investigated using techniques of tissue transplantation, in vitro egg culture and serology. The results suggest that blastocysts display antigens. Prior immunization of allogeneic mouse recipients to cells of the donor strain inhibit the development of eggs transferred to extra-uterine sites but not of ectopic implanted trophoblastic fragments (Simmons and Russell, 1966; Kirby et al., 1966). James (1969) indicated that blastocysts incubated in vitro with immune spleen cells or immune serum do not develop when transferred under the kidney capsule. The nature and time of appearance of antigenicity in the pre-blastocyst egg is unclear. Olds (1968), using fluorescent antibodies on two-celled mouse embryos from which the zona pellucida had been removed claimed to have detected H-2 antigens. However, Sell et al. (see Gardner and Edwards, 1968) were unable to find them on the unimplanted blastocyst. Heyner et al. (1969) reported that naked cleaving mouse eggs from the 8-cell to blastocyst stage were killed when cultured in vitro with allogeneic cytotoxic antibody, but were unaffected when

cultured with antiserum directed specifically against H-2 antigens. These authors suggest that non-H-2 transplantation antigens but not H-2 are present on the pre-blastocyst egg.

The nature and development of antigenic expression in the early embryo will be further discussed in the Introduction to Chapter V.

b) Maternal/foetal exchange

The foetus undoubtedly expresses the antigenic determinants which are the first requirement for a homograft reaction. The second requirement is physical contact between the cells of the foetus and its mother. Trophoblastic cells of foetal origin have been shown to gain access to the maternal system in considerable numbers. Syncytial embryonic trophoblast can be detected in the blood vessels of the uterus of most normal pregnancies in a variety of animals (see Billington, 1970).

Evidence that embryonic cells other than trophoblastic cells cross the placenta in normal pregnancies is limited. Some foetal blood cells enter the human mother through breaks in the blood vessels of the placenta and can be demonstrated in the maternal blood (Lee and Vasquez, 1962). In all likelihood, if red blood cells pass from foetus to mother, some foetal lymphocytes will also do so.

The majority of reports imply that the reverse passage of cells from mother to foetus is rarely a feature of normal pregnancies. In man, despite extensive studies of male abortuses and neonates, maternal cells have been found in only four instances (Taylor and Polani, 1965;

Kodawaki et al., 1965; Lischner et al., 1967; Walknowska et al., 1969). Human maternal cancer cells have occasionally become established in newborn infants (Retik et al., 1962). A report of the passage of large numbers of maternal cells into the mouse foetus (Tuffrey et al., 1969) has not been confirmed (Billington et al., 1969; Seller, 1970).

Although cellular exchange between mother and foetus is limited, the placenta appears to be selectively permeable to immunoglobulins in which placental passage is apparently controlled by the nature of the heavy chain (see Brambell, 1966; Wild, 1966). In some mammals IgG molecules readily cross the placenta whereas IgA molecules, which are similar in size, and IgM molecules, which are larger, do not. In other mammals no antibodies cross the placenta, but there is selective passage across the yolk sac. In the rabbit, maternal antibodies pass through the placenta and home on specific foetal organs without apparent harm (Lanman and Herod, 1965). However, in incompatible blood group matings in man, passage of antibodies may result in haemolytic disease of the newborn.

c) Maternal immunological response to foetal antigens

There is considerable evidence that the mother does become immunized to the foreign antigens of her foetus. Parous female mice have been shown to possess humoral and cellular immunity to the male strain antigens (Goodlin and Herzenberg, 1964; Kaliss and Dagg, 1964; Mishell et al., 1963; Sören, 1967). The immunological consequences of Rh incompatibility between the human mother and her foetus have been well documented (McConnell, 1969).

2. The protection of the foetus from immunological rejection

There is no doubt that the foetus is potentially a 'good' homograft. Antigenic foetal cells gain access to the maternal system which reacts by the production of humoral and cell bound immunity. At least some components of humoral immunity are capable of passing back through the placenta to their foetal targets.

Many hypotheses have been suggested to account for the survival of the foetus despite its similarity to a homograft. These are:

a) the foetus is antigenically immature.
b) the uterus is an immunologically privileged site.
c) the mother is immunologically inert during pregnancy.
d) the foetus and mother are separated by anatomical barriers.
e) the mother produces 'barrier' or enhancing antibody which is capable of protecting the foetus against maternal immune lymphocytes and cytotoxic antibodies.

In the light of more recent experiments several of these theories have been discarded and will be considered only briefly.

a) Little in 1924 suggested that if the foetus does not possess transplantation antigens the problems of immunological incompatibility do not arise. The evidence for the antigenicity of the foetus in normal pregnancy has already been reviewed.
b) The suggestion that foetal survival is due to the immunologically privileged nature of the uterus has been refuted by experiments showing that the uterus is not necessary for normal development (Fawcett, 1960; Kirby, 1960, 1963; McLaren and Tarkowski, 1963;

Billington, 1965), and that it does not protect implanted homografts from rejection (Schlesinger, 1962; Poppa et al., 1964).

c) The rejection of foetal grafts removed from the uterus and placed in the flank muscles of rabbits and rats showed that pregnant females were not immunologically inert during pregnancy, even to their own embryos (Woodruff, 1958). However, there is evidence that animals of several species react less vigorously to homografts during pregnancy. Prolongation of skin homografts occurs in man (Andresen and Monroe, 1962) and in rabbits (Heslop et al., 1954), but has not been detected in cattle (Billingham and Lampkin, 1957). It occurs in the presence of minor histocompatibility differences in mice, not in H-2 incompatible matings (Medawar and Sparrow, 1956; Simmons et al., 1967). The increased secretion of corticosteroid hormones (which have immunosuppressive properties) during pregnancy is thought to be responsible (Medawar, 1953, 1969). Since there is no evidence that pregnancy can weaken a pre-existing state of immunity, it is unlikely that the lower immunological response of pregnancy is an important factor in foetal protection. Billingham et al. (1951) have made the interesting suggestion that hormones secreted by the placenta might act locally to interfere with maternal lymphocyte and foetal cell interactions.

d) Separation of maternal and foetal tissues by anatomical barriers

1. The placental barrier

Close physical connection between mother and foetus is vital to the embryo for the exchange of nutrients and excretory products. This connection is minimized by the provision of separate maternal and foetal circulations in the placenta, which

undoubtedly reduces the immunological contact between the two organisms. The dangers of a common circulation are seen in the situations of 'runting' or transplantation disease (Billingham, 1967). However, it is difficult to see how a lack of blood connections could cause a failure to elicit maternal sensitization or provide a resistence to it. Transplants of the avascular tissue of the epidermis in rabbits and guinea-pigs induce immunity and are rejected in a normal manner (Billingham and Sparrow, 1954).

In 1928 Oettingen and Witebsky were the first to put forward the idea that the placenta could function as a barrier if its trophoblast cells were non-antigenic. This suggestion was made as a result of their findings that human placental villi are deficient in blood group antigens. Medawar (1953) restated the thesis that the presence of a physical barrier between the foetus and the mother might be responsible for foetal survival from the homograft reaction.

The only component of the conceptus that comes into direct contact with maternal tissue after the formation of the placenta is the trophoblast, and this part of the placenta has been shown to persist throughout pregnancy (Bradbury, Billington and Kirby, 1965). The essential role of the trophoblast in the immunological problem of pregnancy has been shown by transplantation studies. Trophoblastic grafts and blastocysts transplanted to extra-uterine sites develop successfully without rejection in mice (Kirby, 1960, 1963; Simmons and Russell, 1962; McLaren and Tarowski, 1963), in rats (Kirby, 1962), in guinea-pigs (Bland and Donovan, 1965) and in hamsters (Billington,

1966). Even xenogeneic grafts grow and proliferate successfully: mouse in rat (Kirby, 1962; Simmons and Russell, 1967b) and mouse in hamster (Billington, 1966). Trophoblastic grafts grow in extra-uterine sites in the presence of a pre-existing immunity in mice while embryonic implants in the same situation are destroyed (Simmons and Russell, 1967a; Kirby et al., 1966)

These experiments indicate that trophoblastic tissue is either non-antigenic in situations where the rest of the embryonic tissue is clearly expressing antigenicity, or is somehow capable of resisting immunological attack.

The failure of the trophoblast to express histocompatibility antigens has been demonstrated serologically in mice, using haemagglutination inhibition techniques and in human trophoblast using immune absorption techniques (Schlesinger, 1964; Seigler and Metzgar, 1970).

Although trophoblast does not express histocompatibility antigens in the normal manner, there is some evidence that it is not entirely devoid of all antigenicity. Simmons and Russell (1967b) have demonstrated species-specific trophoblastic antigens in the rat and mouse. The possession of tissue specific antigens has also been suspected from reports that antibodies to trophoblast appear in the post-partum period in normal human pregnancies and in the serum of women after abortion (Hulka et al., 1963). Anti-trophoblastic antibodies cannot be detected during a normal pregnancy and may only occur after normal or abnormal separation of the placenta.

The mechanism by which the trophoblast fails to express antigens expressed by the rest of the foetal tissue is disputed. Two main hypotheses have been put forward.

In 1964, Kirby et al. postulated that transplantation antigens are present on trophoblastic tissue but their expression is prevented by an extracellular layer which they called the fibrinoid layer. Simmons and Russell (1966), in contrast, believe that trophoblastic cells fail to develop histocompatibility antigens.

Conflicting experimental evidence about the nature and properties of the fibrinoid layer has led to disagreement about its capacity to mask trophoblastic antigenicity. Histochemical and electron-microscopic observations of trophoblastic tissue supported the existence of a 'masked' antigenicity. The physical presence of a darkly staining surface coat of mucoprotein rich in sialic and neuraminic acids was identified around murine trophoblast cells and seemed to form a boundary layer between maternal and foetal tissue (Bradbury et al., 1965). The first appearance of this extracellular layer was found to coincide with the time at which an embryonic implant did not succumb to a pre-existing immunity when transplanted to an extra-uterine site (Potts, 1965). The presence of this extracellular layer has subsequently been identified in the placenta of many mammals, including man (Wynn, 1967; Boyd et al., 1968; Bradbury et al., 1969). However, other workers have failed to detect the presence of an extracellular layer round murine trophoblastic cells (Simmons et al., 1967). It does not appear to be present on rabbit trophoblast (Tai and Halacz, 1967), and in the rat, where fibrinoid is present, it has been reported not to form an intact barrier between trophoblast and decidua (Martinek, 1970).

Experimental evidence supporting the presence of a barrier surface coat surrounding trophoblast cells has come from the observations of Currie and Bagshawe (1967). They showed that when choriocarcinoma cells (malignant trophoblast) were treated with trypsin, and cultivated with allogeneic lymphocytes in vitro, they died, whereas syngeneic tissue survived. This experiment is open to criticism as choriocarcinoma cells were not tested with syngeneic lymphocytes as a control.

Currie and Bagshawe have suggested a possible mechanism by which the fibrinoid barrier could function, from a consideration of the behaviour of cancer cells which appear to be surrounded by a similar substance. They postulate that tumour antigens and trophoblastic antigens escape recognition in the same way, and believe that sensitized lymphocytes, which are known to be negatively charged, could be electrostatically repulsed if a similar negative charge was present on the extracellular layer. Stoward (1968) added some support for the presence of a negative charge on the fibrinoid layer by showing that a high degree of sulphation occurred in the mucoprotein component of fibrinoid material. Sulphation is known to be associated with negatively charged cells, but the possession of such electro-chemical properties by the sialomucins of the extracellular layer has been disputed by Good (1967), who believes that electrostatic repulsion in an aqueous medium would not be possible without damaging the tissues. The nature of the non-antigenicity of the trophoblastic tissue remains to be clarified.

2. Barriers before the formation of the placenta

The establishment of antigenicity in the early embryo has stimulated investigation into the possible mechanisms protecting the unestablished embryo.

Before implantation, the egg is enveloped in the zona pellucida and several investigators have suggested that this membrane might limit immunological contact between the maternal system and the unimplanted egg (Simmons and Russell, 1966, 1967a; Shelesnyak et al., 1967; James, 1969; Heyner et al., 1969). However, in lactational delay, the zona is shed at about the normal time but implantation is successful four days later. In ovariectomized, progesterone-maintained pregnant females, highly immunized to paternal antigens, no effect on blastocyst viability was observed after ten days of zona-free existence (Kirby, 1969b, 1970). The presence of the zona pellucida is, therefore, not essential for embryonic viability and development.

Kirby et al. (1966) have put forward the hypothesis that after the shedding of the zona pellucida at implantation the decidual cell reaction of the uterus might act as an immunological barrier. This subject is discussed in the Introduction to Chapter VI.

e) Tolerance or unresponsiveness induced by "enhancing"antibodies

The most recent theory to account for the success of the foetus as a homograft has been suggested by the finding in mice that multiparity leads to a long lasting, specific tolerance to paternal skin grafts (Woodruff, 1957; Breyere and Barrett, 1960;

Breyere and Burhoe, 1963) despite the presence of humoral and cell-bound immunity to the male strain antigens (Goodlin and Herzenberg, 1964; Kaliss and Dagg, 1964; Mishell et al., 1963; Sören, 1967). On the basis of these observations Kaliss and Dagg (1964) suggested that immunological enhancement "a frustration of both the antigenic stimulus and the host's cellular immune response by humoral antibody" rather than conventional tolerance might account for specific maternal unresponsiveness. The contribution of this phenomenon to foetal protection will be discussed in the introduction to Chapter IV.

3. Evidence for immunological interactions between mother and foetus

A. Studies not involving manipulation of the mother's immunological status

Genetical studies, other than those of parity, have produced only circumstantial evidence of immunological interaction between mother and foetus. Genetic dissimilarity between mother and conceptus may affect many aspects of pregnancy. The mechanisms underlying these phenomena are not understood. Mouse spermatozoa carrying the 't' allete of the tailless (T) locus have an advantage over other spermatozoa (Braden, 1958). In an inbred line of rats, Michie and Anderson (1966) have suggested that continuing heterozygosity at a locus determining transplantation antigens, might be due to a process of differential fertilization.

It has been suggested that in some respects the mother's immune reaction may actually favour the embryo (Kirby et al., 1966). The evidence favouring this suggestion has come from several sources.

In mice a series of crosses showed deficiencies of maternal genotypes, for coat colour and transplantation antigens, among the offspring (Hull, 1964, 1969). Similar results were obtained with transplantation antigens in rats (Palm, 1969). Clarke and Kirby (1966) suggested that the mother's immunological reaction against her heterozygous offspring might cause an increased trophoblastic growth, and so improve their fitness.

F_1 hybrid placentae are usually heavier than inbred placentae (Billington, 1964; McLaren, 1965; McCarthy, 1965). This has usually been attributed to hybrid vigour. In experiments involving egg transfer in mice Billington (1964) found that inbred A_2G eggs transferred to a C57 BL mother gave rise to larger placentae than C57 BL eggs grown in a C57 BL mother. He argued that in this situation hybrid vigour was eliminated and suggested that immunological factors might be involved. However, egg transfer experiments do not exclude the involvement of other phenomena, such as genotypic complementation, in which each genotype compensates for the deficiencies of the other. Furthermore, the size to which a placenta develops is known to be affected by the genotype of the conceptus (McLaren, 1965; McCarthy, 1965).

Support for the hypothesis that immunological interaction might be responsible for the larger size of hybrid placentae came from an investigation into the degree of trophoblastic invasion in extra-uterine sites made within and between strains of mice differing at the H-2 locus. Billington (1965) claimed that trophoblastic transplants showed an increased invasiveness in the testes of allogeneic hosts, compared to those in isogeneic testes. He suggested that

invasiveness was increased when graft and host differed antigenically. If placental weight depended on the degree of trophoblastic invasion, increased invasiveness could explain the larger F_1 placenta found in interstrain matings.

B. Studies involving manipulation of the mother's immunological state

Studies of pregnancy after maternal immunization to paternal antigens have usually produced negative results. The majority of workers have reported that the survival of the foetus is unaffected by the immunological status of the mother (in mice, Mitchison, 1953; Medawar and Sparrow, 1956; Hašková, 1961: in rats, Woodruff, 1958: in rabbits, Heslop et al., 1954; Woodruff, 1958).

Rabbit blastocysts transferred to the uterus of females hyperimmunized to the antigens of both parents survived without apparent harm (Lanman et al., 1962).

Contrary to these findings, studies of James (1965, 1967) have suggested that in mice the immunological state of the mother is an important determinant of foetal and placental growth. Pre-immunization of females to paternal antigens increased the foetal and placental growth and decreased the time of gestation. In females rendered tolerant, foetal and placental weights were reduced and gestation time was increased. These results supported the view that the greater placental weight found in the presence of genetic disparity had an immunological basis. They firmly indicated that immunological reactivity strongly influenced foetal and placental growth.

These considerations led Clarke and Kirby (1966) to postulate that improved foetal growth could provide a selective mechanism capable of maintaining

balanced polymorphism at histocompatibility loci.

4. Nature of the proposed work

During the B.Sc. course in Edinburgh in 1967 I carried out an experiment designed to investigate the nature of trophoblastic growth transplanted to the testis of allogeneic recipients. Trophoblastic tissue was transplanted between and within two strains of mice and the F_1 hybrid. In addition, transfer was made in the presence or absence of prior immunization of the recipient to donor antigens. The published paper resulting from this work (Clarke, 1969) is enclosed in Appendix 1.

In contrast to the results of Billington (1965) I failed to detect any difference in the degree of trophoblastic invasion between isogeneic and allogeneic transfers. I also failed to find any effect of pre-immunization. It appeared that non-specific factors, namely the size of the donor tissue transplanted and the genetic constitution of the receipient testis, determined the extent of trophoblastic growth.

In confirmation of these findings James (unpublished data), using the mouse testis as the graft site, and Koren et al., (1968), using the mouse kidney, have reported an absence of correlation between the extent of trophoblastic invasion and the degree of antigenic difference.

The work described in this thesis arose from the discovery that trophoblastic invasion did not, after all, appear to be influenced by genetic disparity between graft and recipient, or by the immunological status of the recipient, It therefore seemed important to clarify the effects of immunization on placental and foetal growth.

Chapter II describes some experiments showing that, contrary to earlier reports, allogeneic pre-immunization has no effect on placental size, and may reduce foetal weight.

The work in Chapter II led me to investigate further the deleterious effects of allogeneic immunization on the outcome of a pregnancy. Chapter III describes attempts to alter the properties of the placental barrier by the use of enzymes known to affect placental permeability and reputed to disrupt the extracellular layer around trophoblastic cells *in vitro*. It was concluded that the enzyme hyaluronidase, increases early foetal death and that this effect appears to occur only in the presence of allogeneic immunity. Chapter IV describes an experiment on the role of the spleen in foetal protection. The presence of the spleen has been shown to be essential for the induction of "enhancing" antibodies in the protection of tumour homografts. The results suggest that the phenomenon of enhancement may also be involved in the protection of the early foetus.

These investigations led me to consider the immunological factors concerned with protecting the early embryo before the formation of a definitive placenta. Chapter V describes experiments attempting to detect the first appearance of paternal transplantation antigens on the F_1 hybrid embryo. The results of culturing eggs in the presence of antiserum indicate that oviducal hybrid embryos are deficient in paternal antigens. Chapter VI was concerned with the hypothesis that immunological reactions occur between the mother and her foetus at the time of implantation. An investigation was made on the effects of active immunization and of passively transferred isoantisera on the development of the uterine decidual cell reaction at implantation. The

results show that allogeneic immunization to paternal antigens significantly lowers the decidual response. These experiments also give evidence that a particular kind of humoral immunity is responsible for the effect. The effectiveness of passively transferred isoantisera in reducing the decidual cell reaction clearly demonstrates the importance of this humoral antibody factor.

The general implications of the experimental results are discussed in Chapter VII.

Chapter II

The effects of maternal preimmunization on placental and foetal size

The effects of maternal preimmunization on placental and foetal size

Introduction

The work of James (1965, 1967) provided experimental evidence of the theory postulated by Kirby et al. (1966) that the determination of placental size in the presence of genetic disparity had an immunological basis. C57 BL mice preimmunized by two skin grafts and three spleen cell injections from the paternal A_2G strain gave rise to F_1 hybrid offspring which had significantly larger placentae than non-immunized controls. In addition, foetuses were larger from sensitized mothers. When, however, females were rendered tolerant to paternal antigens by neonatal spleen cell injection, foetal and placental weight were decreased. These results suggested that the immunological status of the mother in H_2 incompatible matings was an important determinant of placental and foetal growth. The present experiments were carried out to re-examine the effect of maternal sensitization on placental and foetal weight. A publication reporting these results is enclosed in Appendix 2.3.

Materials and Methods

The first experiment utilized the same strain combination as that used by James (1967). In the second experiment, a different paternal strain was used and an additional group of females was immunized xenogeneically to a Peromyscus species. This latter group was included to investigate the effect of a general non-specific heightening of the immune response, and the effect of stress experienced by the females during the immunization procedure.

Experiment I

C57 BL female mice, aged approximately 6 weeks, were divided into three groups. The first group was immunized against A_2G male mice either with five spleen-cell injections (a half-spleen equivalent each), or five spleen-cell injections and two skin grafts, or two spleen-cell injections and two skin grafts. All treatments were given at fortnightly intervals. Full thickness skin grafts were applied using the method of Gottfried and Padnos (1959). The degree of immunity was measured by the human serum dextran technique (Gorer and Mikulska, 1954). The mice were then weighed, and mated to A_2G males. The second group was left untreated but also mated to A_2G males. The third group was untreated and mated to C57 BL males.

A fourth group of C57 BL female neonatal mice was injected with either 5 to 8 million A_2G spleen cells or with 16 million F_1 C57 BL female x A_2G male spleen cells to render them tolerant to the paternal strain. Injections were made into the anterior facial vein within 24 hr of birth.

All mice were killed on the 18th day of pregnancy (1st day = day of vaginal plug). Embryos and placentae were removed from the uterus, blotted and weighed to the nearest milligram on a torsion balance. The size of a litter (live foetuses) and the number of deaths during early and middle pregnancy ('moles') were recorded. The reciprocal mating, A_2G females x C57 BL males, was not studied.

Experiment II

C57 BL female mice, aged approximately 10 weeks, received three spleen-cell injections (a half-spleen equivalent each), and two skin grafts from either CBA males or from Peromyscus polionotus polionotus (the deer mouse). A control group was not immunized. All females were mated to CBA males and their embryos were recovered as previously described. This experiment was carried out in a different laboratory from that in which Expt. I was conducted and utilized a different population of mice.

Results

Experiment I

The inbred controls had significantly smaller foetuses and placentae than the two outbred groups ($p < 0.001$; see Table 2.1 and Appendix 2.1 for data details); this finding is in agreement with McCarthy (1965) and McLaren (1965). No significant difference was found, however, between the immunized and non-immunized mothers in the weight of the hybrid foetuses and placentae. Nevertheless, immunization appeared to have adverse effects on the outcome of a pregnancy. It significantly decreased the size of a litter ($p < 0.01$) and among those females whose litters contained early and middle deaths ('moles'), there were larger numbers of moles within the immunized mothers ($p < 0.05$, Fisher's exact test). The total number of implantations in a litter (live embryos + moles) was significantly lower in immunized females than in non-immunized or in inbred controls ($p < 0.05$; see Table 2.2). The number of young in a litter is known to affect placental and foetal weights (Healy, McLaren

Table 2.1

Comparison of mean 18th day foetal and placental weights in allogeneically immunized, non-immunized and inbred mice - Experiment I

Group	No. of litters	Mean litter size	Mean foetal wt (mg ± S.E.)	Adjusted* mean foetal wt (mg)	Mean placental wt (mg ± S.E.)	Adjusted* mean placental wt (mg)
Immunized C57 BL x A_2G	25	4.1 ± 0.5	876 ± 23.6	853	145.3 ± 4.6	140.6
Control non-immunized C57 BL x A_2G	12	7.6 ± 0.5	899 ± 10.3	899	140.3 ± 5.5	144.9
Inbred C57 BL x C57 BL	13	6.0 ± 0.7	729 ± 16.9	730	111.6 ± 3.1	112.5

* Individual within-group regression coefficient used to adjust foetal and placental weight means to a constant litter size.

Table 2.2

Comparison of number of early and middle deaths or moles - Experiment I

Group	No. of litters*	Mean litter size	Mean no.** moles/ litter	Mean no. of implantations (embryos + moles)	Females with three or more moles/ litter	Females with < three moles/ litter
Immunized C57 BL x A_2G	34	4.2 ± 0.4	3.4	5.8	11	7
Control non-immunized C57 BL x A_2G	25	6.5 ± 0.5	1.9	7.3	2	9
Inbred C57 BL x C57 BL	14	6.1 ± 0.6	2.5	7.0	2	4

* Litter numbers larger than in Table 2.1 as not all litters used for 18th-day foetal and placental analysis.

** Among females with moles.

and Michie, 1960). Regression analysis allows these weights to be corrected to a standard litter size. Regression of foetal weight on litter size ($b = -14.80$; $0.1 > p > 0.05$) is just below significance but regression of placental weight on litter size is significant ($b = -4.20$; $p = 0.01$). Although the correction for litter size reduces the mean foetal weight of the immunized group where litter sizes were small, the adjusted means of the control and immunized groups are not significantly different ($p > 0.05$). This result is not in agreement with James (1967) who found an increase in placental and foetal weight after maternal immunization. The data in Table 2.1 show, if anything, a decrease in foetal weight.

The degree of immunity of immunized females as measured by the human serum dextran technique was extremely variable and could not be correlated with the mode of immunization. Haemagglutination titres ranged from 1/16 to 1/16,384. Litter sizes, foetal and placental weights and the number of moles in a litter could not be correlated with the rejction times of the second immunizing skin grafts (6.0 ± 2 days), or with the titres of circulating antibody.

Of the forty-eight C57 BL female mice that were neonatally injected with A_2G male spleen cells, all but two developed runt disease. Varying degrees of the typical symptoms (cessation of growth and development, the presence of diarrhoea, sparse hair from about 2 weeks of age) were followed at varying intervals by death. The two survivors received skin grafts at 8 weeks but normal rejection times (10 ± 2 days) showed the absence of tolerance. The sixty-four neonates receiving F_1 (C57 BL female x A_2G male) spleen cells, in an attempt to overcome runt disease, also showed no tolerance. Ten individuals grafted with A_2G male skin at maturity showed rejection times that were normal.

Experiment II

The results (Table 2.3 and 2.4, see Appendix 2.2 for data details) were essentially similar to those of the first experiment. Analysis showed that the mean F_1 foetal weights from mothers specifically pre-immunized to the paternal antigens were, in this case, significantly smaller than those from control non-immunized mothers ($p < 0.02$). Females immunized against the non-specific antigens of Peromyscus polionotus polionotus gave a mean foetal weight intermediate between the allogeneically immunized and non-immunized groups, but was not significantly different from either of them. Placental weights were similar in all three groups. Although the litter sizes and the total number of implantations were lower in the two immunized groups, the differences were not significant. The overall number of moles was too small for a valid comparison to be made (Table 2.4). As in Expt. I, the regression of foetal weight on litter size was not significant ($b = -10.73$; $p > 0.05$) and the regression of placental weight was significant ($b = -2.1$; $p < 0.05$). Correction of foetal and placental weights to a standard litter size of 7.5 reduced the significance of the difference in foetal weight between specifically immunized and non-immunized females ($p = 0.05$).

Discussion

The results of the two experiments indicate that maternal immunization to specific paternal antigens does not enhance placental and foetal growth. Indeed, both experiments show no effect on placental growth but a reduced foetal growth after allogeneic immunization, although this was significant only in Expt. II. These

Table 2.3

Comparison of mean 18th day foetal and placental weights in allogeneically immunized xenogeneically immunized and non-immunized mice - Experiment II

Group	No. of litters	Mean litter size	Mean foetal wt (mg ± S.E.)	Adjusted* mean foetal wt (mg)	Mean placental wt (mg ± S.E.)	Adjusted* mean placental wt (mg)
Allogeneically immunized C57 BL x CBA	6	7.3 ± 0.6	869 ± 7.3	869	122.7 ± 2.6	122.5
Xenogeneically immunized C57 BL x CBA	8	7.2 ± 0.7	890 ± 34.2	884	118.6 ± 4.0	117.5
Control C57 BL x CBA	10	8.1 ± 0.4	915 ± 15.6	918	119.3 ± 2.0	119.6

* Individual within group regression coefficients used to adjust foetal and placental weight means to a constant litter size.

Table 2.4

Number of early and middle deaths or moles - Experiment II

Group	Mean no.* moles/ litter	Mean no. implantations (embryos + moles)	Females with three or more moles/ litter	Females with < three moles/ litter
Allogeneically immunized C57 BL x CBA	2.0	7.7	0	1
Xenogeneically immunized C57 BL x CBA	2.0	8.4	1	4
Control C57 BL x CBA	1.0	8.6	0	4

* Among females with moles

results therefore conflict with those of James (1965, 1967), who found an increase in placental and foetal weight after maternal immunization. Both of the present experiments suggest that immunization reduces litter size, although the effect was significant only in Expt. I. This was not apparent in the experiments of James, who did not include litters containing less than five young, or litters containing moles. The present findings are in agreement with those of Breyere and Sprenger (1969) who reported a decreased litter size at birth in C57 BL females specifically immunized against DBA/2 sarcoma, and mated to DBA/2 males, but no decrease when C57 BL females were immunized non-specifically to C_3H adenocarcinoma. Recently, Boshier and Moriarty (1970), working with sheep, were unable to find any effect of presensitization of the ewe to the mating ram on fertility, fecundity, placental weight or foetal weight. The present findings in Expt. II also indicate that non-specific xenogeneic immunization does not reduce foetal weight significantly, but some effect of stress, or the raising of a general immune response, on foetal weight and litter size cannot as yet be excluded.

Attempts to induce tolerance to A_2G spleen cells in C57 BL female neonates failed because of the occurrence of fatal runt disease (Expt. I). James (personal communication) found only a 5% incidence of runt disease in this strain combination using similar doses of spleen cells.

Billingham and Brent (1959) found the A/C57 BL strain combination the least liable to produce tolerance of many strains tested. They obtained low tolerance in the A into C57 BL combination and in the reciprocal combination (C57 BL into A), they found 100% fatal runt

disease. The A_2G strain was derived about fifty generations ago from the A strain and is unlikely to have acquired many changes at the histocompatibility loci during this time. This has been confirmed by Davies (personal communication) who has typed the histocompatibility loci of the A_2G strain and found them similar to the A strain.

In C57 BL neonates injected with F_1 (C57 BL females x A_2G males), the spleen cells should not be capable of responding to the antigens of the C57BL strain. Nevertheless, difficulty was experienced in bringing these mice through weaning. Billingham and Brent (1959) also report reactions to F_1 cells in the C57/A strain combination.

No satisfactory explanation can be found to account for the difference in the incidence of runt disease between the animals in this study and those of James (1967). The possibility exists that the significantly smaller foetuses and placentae and longer gestation length which James obtained from female mice rendered 'tolerant' to the paternal antigens could have been due in part to subclinical runt disease. Husain and Ketchel (1965) found no influence on the time of parturition in rats which had acquired an immunological tolerance to their hosts.

Additional data on the influence of maternal presensitization to the paternal antigens on these aspects of pregnancy are reported in later chapters. In order to draw conclusions from all the available experiments discussion is referred to Chapter VII, section 1.

Chapter III

The effect of neuraminidase and hyaluronidase on pregnancy in the mouse

The effect of neuraminidase and hyaluronidase on pregnancy in the mouse

Introduction

Several investigators have noted that when the glycosidase enzyme, hyaluronidase, is injected into pregnant rabbits, placental permeability is increased. The nature of the changed relationship is not known but there is no doubt that it has immunological consequences. If skin grafts from female rabbits are transferred to their offspring, rejection is delayed when the mothers have received hyaluronidase during pregnancy. Tolerance to maternal antigens is thought to be induced by the passage of abnormally large numbers of maternal cells to the foetuses.

Nathan, Gonzalez and Miller (1960), working with rabbits showed that in one half of the progeny of mothers injected with hyaluronidase, the survival of maternal grafts was moderately prolonged. In the same species, using higher doses of enzyme, Najarian and Dixon (1963) obtained complete tolerance to maternal skin grafts in 25% of the offspring and partial tolerance in 30%. They also claimed a similar degree of tolerance on the part of the mothers to grafts from their offspring. However, the evidence for the maternal response has not been consistent. Tai and Halasz (1967), although confirming the existence of partial tolerance in the offspring of hyaluronidase treated mothers, found some indication of reduced survival of skin grafts transferred from offspring to mothers. This suggests that sensitization, rather than tolerance, to foetal antigens had occurred.

The action of hyaluronidase on the course of pregnancy and on

the survival of skin grafts exchanged between mother and offspring has not been studied in the mouse.

There are indications that another enzyme, the sialidase neuraminidase could alter the normal immunological relationship between mother and foetus. Currie and Bagshawe (1968) claimed that mouse trophoblastic tissue, cultured in the presence of neuraminidase, was capable of inducing immunity when transplanted to allogeneic recipients. Untreated trophoblast did not do so. Currie and Bagshawe suggested that neuraminidase disrupts the extracellular layer around trophoblastic cells (the periplacental fibrinoid material postulated by Kirby et al., 1964), and exposes the transplantation antigens present on the cell surfaces. The action of neuraminidase on trophoblastic tissue in pregnancy has not, however, been studied.

The experiments reported in this chapter were designed to investigate the effect of neuraminidase and hyaluronidase on pregnancy in the mouse. Particular attention was given to the effects of these enzymes when the mother had been sensitized to paternal antigens.

The first experiments were carried out using Q strain mice. They were designed to determine the tolerance of females and their foetuses to repeated injections of the enzymes, and to discover if the time taken by the offspring to reject maternal skin was altered. Later experiments studied the effects of hyaluronidase and neuraminidase on preimmunized inbred C57 BL mice. It was hoped that by altering placental permeability, the influence of allogeneic immunization on the various parameters of pregnancy (reported in Chapter II) might be clarified.

Experiment I

The effect of neuraminidase and hyaluronidase on the number of live offspring and skin graft survival between mother and offspring in Q strain mice

Materials and Methods

Outbred Q strain virgin female mice approximately 10 weeks old, were mated to inbred CBA males. The pregnant mice were divided into four groups. The first group were given 6 or 8 injections of neuraminidase, in total receiving 16 or 36 international units (I.U.) of enzyme between the 4th or 5th and the 10th or 11th day of pregnancy (vaginal plug = 1st day). The second group were injected with hyaluronidase, receiving a total of 2,000or 4,500 I.U. The third group were given a mixture of both enzymes and the fourth received saline. The enzymes were commercial preparations, supplied by British Drug Houses Ltd. (Neuraminidase from Vibrio cholerae (Mucopolysaccharido N-acetyl neuraminylhydrolase 500 I.U./ml) and hyaluronidase from ovine testes (Hyaluronate lyase, 350-500 I.U./mg). The enzymes were made up in sterile P.B.S. (phosphate-buffered saline) and injections of 0.2 ml were given by the intraperitoneal route.

The number of live offspring (litter size) was recorded at birth. Litters were either allowed to grow to maturity or killed and the female subsequently remated. The remating was carried out to discover if the administration of enzymes during a first pregnancy altered the properties of the placental barrier and allowed the development of an abnormal immunological relationship between mother and foetus. Since immunity takes time to arise, its effects might be detectable only in subsequent pregnancies.

Full thickness skin grafts were transferred when the litters were eight weeks old, either from the mothers to several of their female offspring or from two of the female offspring to their mother. From the 5th postoperative day the grafts were examined daily and the time of rejection was recorded at the first sign of hardening and separation of the donor skin from the graft bed.

Results

1. The number of live offspring born to mothers treated with the enzymes

Repeated injections of enzymes were well tolerated by pregnant females and did not inhibit the normal development of foetuses. Table 3.1 shows that the mean litter sizes of first or second pregnancies were not significantly reduced by any of the enzyme treatments. There is some indication (Table 3.1a) that neuraminidase-treated mothers gave smaller litters than others, but this is not statistically significant ($t = 1.5$; d.f. 26; $P > 0.05$; Student's t test). Details of the data are given in Appendix 3.1.

2. The survival times of skin grafts exchanged between mothers and offspring

The results of skin grafting between mother and offspring do not indicate that there is any significant effect of enzyme treatment on survival time (Table 3.2). Overall rejection times were noticeably short. Whether this was due to the particular strain combination used (outbred Q and Q x CBA F_1 hybrid) is not known. A search of the literature did not reveal any similar studies. The very short survival times were not technical

failures of primary healing; all grafts included in the analysis had healed in satisfactorily prior to rejection. They may be due, in part, to my estimation of rejection time. This was taken at the first sign of hardening of the donor skin, that is the beginning and not the end of rejection.

Offspring born to hyaluronidase treated mothers, seem to have a tendency to reject maternal skin a little slower than offspring carrying skin grafts from mothers not treated with hyaluronidase. There are also indications that the maternal response to skin grafts from their offspring in hyaluronidase treated mothers is slightly faster than in mothers which have not received the enzyme (Table 3.2a - details in Appendix 3.1). The data however, are not sufficient to show any but a large alteration of rejection time. It is not possible to know if the slight differences in response indicate the presence of immunity in mothers and tolerance in the offspring. Neuraminidase treatment does not follow this pattern, if anything, its administration slightly quickens rejection time in both mothers and progeny.

Table 3.1

The effects of neuraminidase and hyaluronidase on litter size at birth

Treatment	Total enzyme dose (I.U.)	1st Pregnancy		2nd Pregnancy	
		Mice No.	Mean Litter Size ± s.e.	Mice No.	Mean Litter Size ± s.e.
Neuraminidase	16 or 36	11	9.3 ± 1.0	5	6.4 ± 1.5
Hyaluronidase	2,000 or 4,500	4	10.8 ± 1.4	3	11.3 ± 1.9
Neuraminidase and Hyaluronidase	16 and 2,000	8	9.3 ± 1.0	3	9.7 ± 3.5
Saline	-	5	11.8 ± 2.0	-	NT

NT - not tested

Table 3.1a

The effect of neuraminidase treatment compared to other treatments on litter size

Treatment	1st Pregnancy		2nd Pregnancy	
	Mice No.	Mean Litter Size (± s.e.)	Mice No.	Mean Litter Size (± s.e.)
Neuraminidase	19	9.3 ± 0.7	8	7.6 ± 1.58
Non neuraminidase	9	11.3 ± 1.2	3	11.3 ± 1.9

Table 3.2

The effect of neuraminidase and hyaluronidase on the survival time (days) of skin grafts exchanged between mother and offspring

Group	Neuraminidase	Hyaluronidase	Neuraminidase and Hyaluronidase	Saline
Mother to Offspring	7, 6, 5, 7	7, 5, 5, 7, 8, 9, 7, 7	8, 8, 7, 7	5, 8, 7
(Average)	(6.3)	(6.9)	(7.5)	(6.7)
Offspring to Mother	7, 6, 6,	5	6, 6	7, 8, 8 9, 7, 9
(Average)	(6.3)	(5.0)	(6.0)	(8.0)

Table 3.2a

The effect of hyaluronidase on the survival time (days) compared to survival time in other groups

Group	Hyaluronidase	Non-Hyaluronidase
Mother to Offspring	7, 5, 5, 7, 8, 9, 7, 7, 8, 8, 7, 7	7, 6, 5, 7, 5, 8, 7
(Average)	(7.1)	(6.4)
Offspring to Mother	5, 6, 6	7, 6, 6, 7, 8, 8, 9, 7, 9
(Average)	(5.7)	(7.4)

Experiment II

The effect of neuraminidase and hyaluronidase on pregnancy in allogeneically preimmunized mice

Materials and Methods

C57 BL female mice approximately 6 weeks old, were immunized to paternal strain antigens with two full thickness skin grafts followed by three spleen cell injections ($\frac{1}{2}$ spleen equivalent each) from CBA male mice at fortnightly intervals. This group, and a control group of similar age were mated to CBA males. Both groups were given daily intraperitoneal injections (from the 5th day to the 15th day of pregnancy) of a mixture of neuraminidase and hyaluronidase. The enzymes were made up in sterile PBS and were given in 0.2 ml doses. The mice received a total of 90 I.U. of neuraminidase and 2,600 I.U. of hyaluronidase. On the 18th day of pregnancy (1st day = vaginal plug), the animals were killed and the following measurements were recorded; foetal weights, placental weights, litter sizes (the numbers of live embryos), the numbers of dead embryos (moles) and the implantation numbers (live and dead embryos).

Moles were classified into early, middle and late foetal deaths using the following criteria:

a) Early moles - foetal deaths occurring early in pregnancy and forming small nodules with no distinguishable foetal form and no division into foetal and placental tissue.

b) Middle moles - deaths occurring around $12\frac{1}{2}$ to $13\frac{1}{2}$ days of pregnancy and forming larger tissue masses with indications of foetal form e.g. some eye and tail features. Foetal and placental components are separate.

c) Late moles - deaths occurring in the last third of pregnancy; fully-formed embryos and placentae.

Statistical analysis of results

Foetal and placental weights were compared using the regressions of foetal and placental weight on litter size (Healey, McLaren and Michie, 1960). Litter size and implantation number were compared using the Student t test or the analysis of variance. Heterogeneity χ^2, and Fisher's exact test was used for statistical comparison of the incidence (%) of females who had moles and for the mean number of total moles (early, middle and late), and middle moles only.

Results

The mean foetal weights, placental weights and litter sizes are given in Table 3.3. Further details about the data can be found in Appendix 3.3a. The regression of foetal weight on litter size was not significant ($b = 6.88 \pm 10.71$; $p > 0.05$). When the mean foetal weights were corrected for litter size, the difference between the immunized and the non-immunized groups fell just short of statistical significance ($F = 4.06$ d.f. 1 and 15; $0.10 > p > 0.05$). When this result is compared with the results reported in the previous chapter, there is nothing to suggest that the enzymes have altered the basic pattern of response to immunization. The foetal weights are, however, considerably lower than those found in the earlier experiments, suggesting that the enzymes have 'non specifically' lowered the foetal weights in both groups.

The regression of placental weight on litter size was negative but not significant ($b = -1.12 \pm 0.82$; $p > 0.05$). When the placental weights were corrected for litter size, there was no evidence of any difference between the immunized and the control groups. The placental weights do not differ from those found in earlier experiments without enzyme treatment.

The mean litter size and the mean number of implantations were lower in the immunized group, but not significantly so. There is also some suggestion that the proportion of females containing moles is higher in the immunized group, but again the difference is not significant. The suggestion is strongest when only 'middle' moles are considered (Table 3.3.a). The results are similar to those obtained in earlier experiments without enzyme treatment.

Table 3.3

Comparison of mean 18th day foetal and placental weights in allogeneically immunized and non-immunized females after treatment with neuraminidase and hyaluronidase.

Group	No. of Litters	Mean litter size (± s.e.)	Mean foetal wt. (mg ± s.e.)	Adjusted mean foetal wt. (mg)	Mean placental wt. (mg + s.e.)	Adjusted mean placental wt.(mg)
Allogeneically immunized (+ Hyal. + Neur.) A	9	5.2 ± 0.9	706 ± 42	712	124.5 ± 3.6	123.5
Non-immunized (+ Hyal. + Neur.) B	9	7.0 ± 0.8	832 ± 31	826	120.6 ± 2.1	121.6

Table 3.3a

The effect of enzyme injection on the incidence and numbers of foetal death (moles) and the numbers of implantations in allogeneically immunized and non-immunized females.

Group	% of females containing moles (middle only)	Mean No. Moles/litter		Mean No. of implantations (embryos+ moles)	Females with three or more moles/litter	Females with < three moles/ litter
		Total*	Middle**			
A Allogeneically immunized (+ hyal. + neur.)	67 (44)	3.0	2.3	7.2	4	5
B Non-immunized (+ hyal. + neur.)	56 (22)	2.2	3.0	8.2	1	8

* among females with moles.

** among females with middle moles.

Experiment III

The effect of hyaluronidase on pregnancy in allogeneically and xenogeneically immunized mice

This experiment reinvestigated the effect of hyaluronidase injections on the pregnancy of allogeneically immunized mice.

Materials and Methods

Five groups of C57 BL female mice were used. They were either preimmunized allogeneically to the antigens of the paternal strain, xenogeneically to Peromyscus antigens or left unimmunized. The preimmunized animals were either injected with a total of 12,400 I.U. of hyaluronidase or with saline daily from the 5th day to the 15th day of pregnancy. Unimmunized mice were injected with saline.

The methods of preimmunization, enzyme injection, the recovery of foetuses and placentae, classification of moles and statistical analysis were as previously described in the last experiment. In addition, middle moles were weighed (to the nearest mg) in order to determine the approximate time of death.

Results

Table 3.4 summarizes the foetal weights, placental weights, litter sizes and implantation numbers in the five groups of mice. Individual data are recorded in Appendix 3.3.

The regression of foetal weight on litter size was negative but not significant ($b = -5.89 \pm 3.87$; $p > 0.05$). There was a significant heterogeneity in adjusted mean foetal weight ($F = 2.99$; d.f. 4 and 42; $p < 0.05$). This heterogeneity was removed when control data were

excluded from the analysis. Between the other groups, foetal weights were homogeneous ($F = 1.55$; d.f. 3 and 4; $p > 0.05$). The adjusted mean foetal weights in both allogeneically immunized groups were significantly lower than those of the control weights ($F = 7.2$; d.f. 1 and 16; $p < 0.05$; and $F = 6.3$; d.f. 1 and 14; $p < 0.05$ respectively). Xenogeneic immunization in the presence of hyaluronidase also gives a significantly lower foetal weight than that of the control ($F = 9.8$; d.f. 1 and 15; $p < 0.01$).

Although the difference between them is not significant, the group of mice xenogeneically immunized and treated with hyaluronidase gave a lower foetal weight than similarly immunized saline injected animals. There is no similar depression among the allogeneically immunized groups.

The regression of placental weight on litter size was significant ($b = -2.33 \pm 0.67$; $p < 0.05$). Adjusted mean placental weights were similar in all groups. There were no significant differences in mean litter size. This was also true of implantation numbers, with the exception of the xenogeneically immunized mice injected with saline (Table 3.4a). This group showed a significantly lower number of implantations than any other ($F = 4.0$; d.f. 4 and 43; $p < 0.01$). Since the tendency is not found in the other xenogeneically immunized group, it is difficult to interpret.

There was no overall heterogeneity between the groups in the proportion of females showing moles or in the mean number of moles they possessed (Table 3.4a). However, individual comparison of the allogeneically immunized group injected with hyaluronidase, with that

Table 3.4

The comparison of mean foetal and placental weights on the 18th day in allogeneically immunized, xenogeneically immunized and non-immunized C57 BL mothers with or without hyaluronidase.

Group	No. of litters	Mean litter size (± s.e.)	Mean foetal wt. (mg ± s.e.)	Adjusted mean foetal wt. (mg)	Mean placental wt. (mg + s.e.)	Adjusted mean placental wt.(mg)
A Allogeneically Immunized + Saline	8	8.0 ± 0.4	859 ± 12	864	114.3 ± 2.4	112.6
B Xenogeneically Immunized + Saline	12	6.3 ± 0.7	911 ± 21	906	119.6 ± 4.5	115.3
C Allogeneically Immunized + Hyal.	10	6.8 ± 0.7	873 ± 17	871	115.7 ± 2.7	115.4
D Xenogeneically Immunized + Hyal.	9	8.2 ± 0.9	852 ± 16	858	114.2 ± 2.2	115.6
E Non-immunized + Saline	9	6.9 ± 0.3	933 ± 16	931	112.2 ± 3.2	111.5

Table 3.4a

The effect of hyaluronidase on the incidence and numbers of foetal deaths (moles) and the numbers of implantations in allogeneically immunized, xenogeneically immunized and non-immunized females.

Group	% of females with moles (middle only)	Mean No. Moles/litter		Mean No. of implantations (embryos + moles)	Mean total wt. middle moles (mg ± s.e.)	Females with three or more Moles/litter	Females with < three moles/ litter
		Total*	Middles**				
A Allogeneically immunized (+ saline)	25 (13)	2.0	2.0	8.3	57.9 ± 0.9	0	8
B Xenogeneically immunized (+ saline)	50 (25)	1.0	1.0	6.9	73.4 ± 10.5	0	12
C Allogeneically immunized (+ Hyal.)	80 (50)	3.4	3.4	9.5	94.3 ± 13.2	5	5
D Xenogeneically immunized (+ Hyal.)	67 (22)	2.0	3.0	9.6	74.2 ± 0.3	2	7
E non-immunized (+ saline)	67 (11)	2.0	1.0	8.6	26.3	2	7

* among females with moles

** among females with middle moles

similarly immunized but injected with saline, showed the former group had both a significantly higher percentage of females which had moles ($p < 0.05$) and a significantly higher mean number of moles ($p < 0.05$).

Early moles in all groups had a similar appearance and size but middle moles showed large individual variation. Table 3.4a records the mean weight of middle moles in each group. The mean weight in the hyaluronidase treated allogeneically immunized group was larger than that found in the other groups. Statistical analysis was not possible as in some groups so few females had middle moles.

General Discussion

There is no evidence that enzyme treatment enhances the effects of allogeneic immunity in depressing foetal weight. However, it appears to have a tendency to reduce foetal weight in the xenogeneically immunized group. The lack of effect on allogeneically immunized females may be due to those latter weights being already depressed by allogeneic immunity.

Hyaluronidase has an effect on foetal death which appears to be specific to the presence of allogeneic immunity. It increases both the percentage of females that possess moles and the number of moles they have. In addition the middle moles are considerably heavier than those of other groups. If the weight of a mole is an indication of the time during pregnancy at which foetal death occurs, it would suggest a small percentage of foetuses are dying at a later stage than normal.

In a study to determine whether hyaluronidase would alter placental permeability so as to allow maternal leukaemic cells to cross to the foetuses of pregnant female mice, Loewenstein, Hughes, Hofer and Ketchel (1971), reported foetal death from leukaemia in 3 out of 20 foetuses. The absence of leukaemia in the litter mates indicated that cell passage from hyaluronidase treated mothers was not a general phenomenon but occurred to a limited extent. It is possible that the increased incidence of foetal death in allogeneically immunized mothers treated with hyaluronidase is the result of a similar phenomenon. Alteration of placental permeability in the presence of a maternal immunity to paternal antigens leads to foetal death in a small percentage of the litter. This damage is represented,

on the 18th day of pregnancy, by an increased number and incidence of moles. Whether the damage is caused by an increase in the passage of maternal antibodies or to an increased exchange of foetal and/or maternal cells cannot be distinguished by those experiments.

Chapter IV

The effects of splenectomy on pregnancy in allogeneically immunized and non-immunized C57 BL mice.

The effects of splenectomy on pregnancy in allogeneically immunized and non-immunized C57 BL mice

Introduction

Investigations into the immunological state of the mother during and after pregnancy have demonstrated that, although immunologically competent, she shows a degree of partial unresponsiveness to foetal and paternal skin grafts (Woodruff, 1957; Breyere and Barratt, 1960). A similar state of partial unresponsiveness has been found to occur following immunization against certain tumours. In these circumstances, tumour graft survival appears to be increased by immunization, and the phenomenon has been termed immunological enhancement (Kaliss, 1957; 1958; Moller, 1963b; 1965; Hellström and Hellström, 1969).

The parallels between the two phenomena have been emphasized by Kaliss and Dagg (1964). Immunological enhancement of tumour homografts is apparently mediated by circulating 'barrier' antibodies in the presence of immune lymphocytes (Kaliss, 1957), while foetal survival also occurs in the presence of antifoetal antibodies (Lanman and Herod, 1965) and immune lymphocytes (Sören, 1967). Tumour enhancement only occurs with tumours which differ from the host with respect to the H-2 locus. These tumours have surface antigens in a low concentration (Moller, 1963b) and are analogous to foetal trophoblast, which is similarly weakly antigenic.

Although immunological enhancement has only been adequately demonstrated in response to tumour proliferation, experimental evidence is available to suggest that the phenomena might occur in the foetal-maternal relationship. In this situation it is supposed that in the presence of circulating antifoetal 'barrier' antibodies, exposed antigenic sites on trophoblast cells become coated by these antibodies

and thereby protected from attack by sensitized maternal lymphocytes.

Enhancing antibodies produced in response to the presence of a tumour in an allogeneic host can be passively transferred by iso-antisera (Kaliss, 1957; 1958). This property of passive transfer distinguishes enhancement from conventional tolerance. There is no direct or conclusive evidence that maternal unresponsiveness to paternal skin grafts can be passively transferred. Initial attempts to demonstrate such a transfer, using serum from multiparous females, were not successful (Kaliss and Dagg, 1964). Pregnancy haemagglutinins, however, have a short half-life on transfer (Rubinstein and Kaliss, 1964) and, in a similar study with more extensive immunization, Currie (1969) claims to have passively transferred enhancement to allogeneic tumours and skin grafts by the injection of allogeneic pregnant serum.

Serum taken from pregnant mice has been shown to be capable of protecting foetal cells in vitro from attack by immune lymphocytes (Hellström, Hellström and Brawn, 1969). These workers have shown that lymphocytes from mice carrying antigenically foreign foetuses inhibit the growth of foetal cells of the same genetic type. When serum from the pregnant mice was added to the cultures the cells remained viable. The authors point out that it cannot be concluded that the protective serum factor is an immunoglobulin. However, in tumour systems, a similarly acting factor, which results in protection from immune lymphocytes, was demonstrated to be a 7S immunoglobulin (Hellström, Hellström and Pierce, 1968; Hellström and Hellström, 1969).

Evaluation of the mechanisms of maternal host unreactivity in pregnancy has involved attempts to break the 'enhancement' and leave the embryo relatively unprotected. Avery and Hunt (1968) have shown that

the nature of the maternal response to a post-partum skin graft after an inter-strain pregnancy may depend on the route of administration and dose of the immunizing stimulus given during pregnancy. Thus, the low doses of antigens received by the mother from the foetal circulation might induce enhancement while the larger doses of antigens given in pre-immunization experiments might result in sensitization. Halpern and his colleagues (1963) found that non-specific stimulants of cellular immunity overcome the enhancement of tumours, and Currie (1969) found that foetal reabsorptions increased in inter-strain pregnancies when female mice were given several injections of the adjuvant Corynebacterium parvum. Currie (1969), however, using large doses of paternal spleen cells, found that immunization did not affect litter size or the number of implantation sites. When doses of paternal tumour cells were used for pre-immunization, foetal death rate tended to be increased.

The kinetics of immunological enhancement are uncertain and many of these attempts to overcome specific unreactivity in pregnancy may have used inappropriate conditions. However, it is clear that the presence of enhancement must depend on some humoral balance between enhancing antibodies and the cytotoxic antibodies involved with the immune lymphocytes in sensitization.

The role of the spleen has been shown by several workers to be critical in the induction of tumour enhancement. Removal of the spleen in mice inhibits the growth of incompatible tumours (Prehn, 1959; Old, Clarke, Benacerraf and Stockert, 1962; Möller, 1965; Ferrer, 1968a). Since the spleen is an important source of humoral antibody (Rowley, 1950; Adler, 1965) the idea has been advanced that the increased resistance of splenectomized mice to tumour rejection is due to the removal of a major source of enhancing antibody (Old et al., 1962). The growth of

tumour can be experimentally increased in mice previously implanted with a graft of the same tumour (Ferrer, 1968a). The effect is specific and is significantly reduced in splenectomized animals. Moreover, serum from mice splenectomized prior to immunization with tumour is significantly less effective in producing passive enhancement of the tumour than serum from controls (Ferrer, 1968b).

The experiment reported here is an investigation into a possible role of the spleen in foetal protection. It was hoped that the combination of splenectomy and immunization would lead to sensitization of the mother without the protective presence of enhancing antibodies.

Materials and Methods

Four groups of C57 BL mice, of similar age were used. Group A and Group B were splenectomized. On the following day Group A and Group C were immunized to the paternal strain, receiving one skin graft and one spleen cell injection ($\frac{1}{2}$ spleen equivalent each) from CBA males. The fourth group (D) was neither splenectomized nor immunized. All mice were mated to CBA males. The methods of immunization, of the recovery of foetuses and placentas on the 18th day of pregnancy, and of the statistical analysis have been described in earlier chapters.

Results

The results suggest that splenectomy does not affect foetal weight, placental weight, litter size or implantation number whether in the presence or absence of immunity. Foetal mortality seems to be unaffected by splenectomy alone but is clearly increased by splenectomy in the presence of immunity.

Table 4.1 records the mean foetal weights, placental weights and litter sizes in the four groups. The regression of foetal weight on litter size was negative but not significant ($b = -7.75 \pm 4.1$; $p > 0.05$). Mean foetal weights adjusted for litter size were not significantly heterogeneous. The group of mice receiving immunization alone, as in earlier experiments, showed a tendency to have lower foetal weights than the other groups. Immunized mice which had also been splenectomized did not show this tendency.

The regression of placental weight on litter size was significantly negative ($b = -2.77 \pm 0.73$; $p < 0.05$. Mean adjusted placental weights were similar in all groups. Litter size and implantation number were also homogeneous (Table 4.2).

The incidence of foetal death is shown in Table 4.2. The percentage of females showing moles appears to be greatly increased by splenectomy in immunized mice.

The heterogeneity between groups is clearly significant ($\chi^2_{(3)} = 11.85$; $p < 0.01$) and comparisons between individual pairs show that this heterogeneity arises from the fact that Group A differs significantly from Groups B and D ($p = 0.025$), and from Group C ($p = 0.01$). When, however, the mean number of moles per female was examined, there were no significant differences between the groups. The division of foetal deaths into early, middle, and late was not appropriate in this series because, with one exception, all deaths were early.

There was no difference in the time of skin graft rejection between groups (8 ± 2 days). A negative correlation exists in

Table 4.1

Comparison of mean 18th day foetal and placental weights in allogeneically immunized and non-immunized C57 BL mice with or without splenectomy.

Group	No. of Litters	Mean litter size (± s.e.)	Mean foetal wt. (mg ± s.e.)	Adjusted mean foetal wt (mg)	Mean placental wt. (mg ± s.e.)	Adjusted mean placental wt.(mg)
A Immunization and Splenectomy	14	6.4 ± 0.7	916 ± 19	912	109.0 ± 1.8	107.8
B Splenectomy only	11	7.2 ± 0.5	922 ± 20	925	101.1 ± 3.9	102.1
C Immunization only	14	7.6 ± 0.4	882 ± 12	889	102.9 ± 2.8	105.2
D Controls	13	6.1 ± 0.6	913 ± 12.9	908	111.5 ± 4.1	109.4

Table 4.2

The effect of splenectomy on the numbers of implantations, and the numbers of foetal deaths (moles) in allogeneically immunized and non-immunized females.

Group	% of females containing moles	Mean no. * moles/litter	Mean no. of implantations (embryos + moles)	Females with three or more moles/litter	Females with < three moles/litter
A Immunization and Splenectomy	78.6	1.8	7.8	2	12
B Splenectomy only	27.3	1.7	7.6	1	10
C Immunization only	21.4	1.0	7.9	0	14
D Controls	30.8	2.5	6.8	3	10

* Among females with moles

Group A between the number of days from splenectomy (and immunization) to the onset of pregnancy (day of plug) with the number of foetal deaths. The correlation was not, however, significant.

Discussion

On the basis of the present experiments, there appears to be no direct correspondence between the role of the spleen in tumour systems and its role in pregnancy. In the tumour system the growth of incompatible tumours is increased by prior immunization of the recipients with a similar graft. This increased growth is prevented by the removal of the spleen.

The removal of the spleen from females preimmunized to maternal strain antigens does not significantly affect the weight of hybrid 18th day foetuses, but does increase the incidence of early foetal death. This result suggests that the spleen (and enhancing antibodies) might play a role in protecting the early embryo (perhaps before the establishment of trophoblastic barriers). It does not, however, appear to affect later development. It is, of course, possible that after splenectomy other organs of the immune system take over the role of the spleen.

Although mean foetal weights did not differ significantly between the groups there is a tendency towards higher foetal weights in splenectomized animals. This is true of comparisons within both the immunized and the non-immunized groups. If this observation were to be repeated and found to be genuine it would suggest that the spleen has an influence on general foetal growth. One could speculate that the apparent lack of effect of immunization on foetal weight after

splenectomy was due to the lowered immune response to injected antigen. The removal of the spleen would then effectively reduce the effects of preimmunization.

Under the conditions of this preliminary investigation the phenomenon of enhancement would appear to be involved in the protection of the early foetus.

Chapter V

Studies on the expression of transplantation antigens in early development

Studies on the expression of transplantation antigens in early development

Introduction

The existence of transplantation antigens in the early mammalian embryo has been well established. However, the time of development, the first appearance and the nature of paternal antigenic expression has not yet been clarified. From the immunological point of view the expression of paternal antigens is more important than maternal antigenic expression.

A recent study has shown that, by the 6th day of pregnancy in the mouse, the hybrid pattern of glucose phophate isomerase-1 is discernible (Chapman, Whitten and Ruddle, 1971). This locus is, therefore transcribed and translated before the 5th day. Thus at least part of the paternal contribution to the genome of the egg is active at this early stage.

The experiments reported in this chapter were undertaken to determine whether paternal transplantation antigens could be detected on the surface of early hybrid eggs. Zona-pellucida-free tubal eggs were cultured in the presence of antibody, prepared against tissues of the paternal inbred strain.

Experiments I, II and III were preliminary studies prior to an experiment (IV) on hybrid eggs. Experiments I and II were designed to determine if the proposed culture system was capable of supporting egg development. Experiment I tested the viability of hybrid eggs in the presence of guinea pig serum (GPS) and mouse serum. Experiment II involved the use of various commercial preparations of GPS to discover

a source which was not toxic to eggs. Experiment III was concerned with the effects of anti-CBA serum on CBA eggs, and set out to determine if the system of culture was capable of showing immunological effects. The comparative study on the development of C57 BL x CBA F_1 hybrid eggs in the presence of anti-CBA serum is reported in Experiment IV.

General Material and Methods

A. In vitro Culture

Eggs were collected on the afternoon of the third day of pregnancy (first day = vaginal plug) by flushing the oviducts with phosphate-buffered saline (PBS) to obtain 8-cell embryos. They were cultured in microdrops under liquid paraffin (Brinster, 1963), using the medium of Brinster (1965). The cultures were maintained for several days at 37°C in an atmosphere of 5% CO_2 in air. The eggs collected from each female were randomized between treatments and placed singly in culture drops to avoid spontaneous fusion. Culture drops were made up fresh each day and were allowed to equilibrate for two hours in the incubator before eggs were added. Guinea pig serum (GPS) was used as a source of complement for the initiation of cytotoxic antigen-antibody reaction. The appropriate experimental antiserum or control serum was added, with GPS, to Brinsters medium in making up the culture media. The removal of the zona pellucida was carried out by placing the eggs in dialysed pronase solution (0.5% in phosphate-buffered saline with 10 mg/ml of polyvinyl pyrrolidone) for about ten minutes (Mintz, 1967). Eggs were removed when the zona pellucida swelled and thinned and were washed in two rinses of PBS before being placed in the appropriate culture drops. Eggs which did not lose the zona remnant in the rinses were discarded. As far as possible, eggs collected each day were distributed equally between all treatments, so that comparisons of subsequent development would be as free from 'day-to-day' variables as possible.

B. Preparations of Antiserum and Estimation of Titre

Antiserum was collected from females which had been immunized by two full thickness skin grafts and three spleen cell injections (half

spleen equivalent each) from the paternal strain. Immunization was given at fortnightly intervals. Collection of antiserum was made one week after the last injection and the serum was stored at -30°C until required. Antiserum from each female was kept separately and in most cases was tested before use for haemagglutination titre using the human serum dextran method (Gorer and Mikulska, 1954). Estimations of antibody titre were made in an attempt to determine if antisera of high titre would have a greater effect on growth and development than those of a low titre. Titres of antiserum varied from 32^{-1} to 512^{-1}. In experiments for which decomplemented antiserum was required, the antiserum was heated in a water bath at 56°C for thirty minutes before use. Control non-immune serum was collected from females of a similar age to those immunized, and stored at -30°C until use.

C. Criteria for assessing Development of Eggs

Eggs were examined at least once a day for several days. After twenty four hours of culture the presence of dead eggs and morulae was recorded. After forty eight hours the formation of blastocysts was similarly recorded, and subsequent blastocyst attachment and the presence of trophoblastic outgrowth were followed for several more days. In later experiments two additional criteria were added, in the light of knowledge gained in the first experiments: (1) The time at which cavitation occurred at the beginning of blastocyst development, and (2) The normality or abnormality of blastocysts, classified macroscopically. Blastocysts were counted as abnormal if there was permanent formation of more than one cavity, if only a proportion of the cells had contributed to the blastocyst or if large extrusions of dead cells were present on the surface of the embryo. Eggs were

classified as normal if at any time they formed fully expanded blastocysts. Photographs of normal and abnormal blastocysts were taken.

Experimental days in which there was no development to the blastocyst stage in any treatment group were not included in the data.

D. Analysis of Results

Each experiment involved the setting up of several cultures of eggs. These cultures were established on different days and consequently there was inevitably variation between cultures, within experiments. The figures in the Tables represent the sums of all cultures for each experiment. The individual figures for each culture are given in Appendix 5.

The individual, rather than the overall, figures were used for statistical analysis. This enabled direct comparisons to be made between the developmental capacities of eggs grown in different media, but otherwise subjected to the same conditions of culture. The Binomial Test and the Mann-Whitney U Test (Siegel, 1956) applied to the percentages, allowed the results of individual comparisons to be combined. The conventional χ^2 was not used because the numbers of eggs cultured on each day were too small, and a test of the sums of cultures is not valid because it combines heterogeneous data.

Weighted regression analyses were employed to discover if there was any relationship between the levels of antibody in a given antiserum, and the pattern of egg development. The regressions on antibody titre of the following variables were examined: percentage of eggs forming blastocysts; percentage of blastocysts showing normal development;

and percentage of normal blastocysts developing into trophoblastic outgrowth. In each case the percentages were transformed into angles and weighted by the number of observations in the sample (Fisher and Yates, 1963).

Individual Materials and Methods

Experiment I

The culture of C57 BL x CBA F_1 eggs to test the viability of eggs in a medium containing antiserum and GPS

A preliminary experiment was carried out to test the viability of C57 BL x CBA F_1 hybrid eggs in the proposed culture system of antiserum and complement, and to assess the optimum conditions for culture.

Eight-cell eggs, with or without the zona pellucida, were cultured in 1% of either C57 BL anti-CBA serum or C57 BL anti-*Peromyscus* serum with 25%, 10% or in the absence of GPS (Preserved guinea pig serum, Wellcome Labs.). The group of eggs cultured in C57 BL anti-*Peromyscus* serum was included to detect any non-specific effects of antiserum on egg development. A group cultured in Brinster's medium alone was also included.

Experiment II

The culture of outbred (Q) eggs to investigate the relative toxicity of various sources of complement

Because the GPS was toxic, the conditions in Experiment I were evidently unsatisfactory for either a comparative study of development between eggs cultured in antiserum and control serum or an investigation of the effects of different antisera. It was therefore necessary to find a source of complement that did not kill eggs.

Culture drops were set up containing either C57 BL anti-CBA serum (immune serum), or non-immune Q strain serum, in the presence or absence of three types of GPS 1) preserved GPS (Wellcome Labs.), 2) freeze-dried GPS (Wellcome Labs.) or 3) fresh GPS. Sera and GPS were either

untreated or were heat inactivated for thirty minutes at 56°C to remove complement. All eggs were treated with pronase to remove the zona pellucida. Culture drops using preserved GPS were set up in the following combinations in an attempt to determine the cause of toxicity:

1. Culture medium only.
2. Inactivated immune serum and GPS.
3. Inactivated immune serum and inactivated GPS.
4. Immune serum and GPS.
5. Immune serum and inactivated GPS.
6. Immune serum only.
7. GPS only.
8. Inactivated immune serum only.
9. Inactivated Q serum and GPS.
10. Q serum only.

Experiment III

In vitro culture of inbred CBA eggs in the presence of C57 BL anti-CBA serum

CBA, zona-pellucida-free, 8-cell eggs were cultured in Brinster's medium containing either 10% C57 BL serum, or 10% immune serum. 10% fresh GPS was used as the complement source. Antiserum was heat inactivated at 56°C for thirty minutes to remove mouse complement. Fresh GPS was untreated. The titre of antibody in the antisera was recorded.

Experiment IV

In vitro culture of C57 BL x CBA F_1 eggs in the presence of C57 BL anti-CBA serum

C57 BL x CBA F_1 hybrid, zona-pellucida-free, 8-cell eggs were

cultured in Brinster's medium containing 10% C57 BL anti-CBA serum and either 1% (Series I) or 10% (Series II) fresh GPS. Inbred C57 BL and inbred CBA eggs were cultured in the same medium. Antisera was heat inactivated at 56°C for thirty minutes to remove mouse complement. Fresh GPS was untreated. The titre of antibody in the antisera was recorded in Series II.

Results

Experiment I

Table 5.1 shows that GPS was toxic to all developing eggs. In the presence of 25% GPS none of the eggs cleaved, and the zonas (on eggs that possessed them) were swollen. 10% GPS allowed some development of 'zona intact' eggs (to the morula stage but rarely beyond), but there was no cleavage of 'zona free' eggs. The antisera were also toxic, but to a much smaller degree. In the absence of GPS, anti-CBA serum had little effect on 'zona intact' eggs (83% of them formed blastocysts) but was significantly toxic to 'zona free' eggs (14% blastocysts). Anti-Peromyscus serum appeared to be toxic to both types of egg. The greater toxicity of the latter serum is difficult to understand, but its higher titre may have revealed the effects of cross-reacting antibodies.

Experiment II

The results shown in Table 5.2 show the preserved GPS is toxic to egg development. The percentage of eggs developing to the blastocyst stage is approximately halved in the presence of 10% GPS. Heat inactivation largely removes the toxic effects, the percentage of blastocysts in this group being comparable to that found in the control group. Of the 54 eggs cultured in the presence of untreated GPS, 14 (25.9%) reached the blastocyst stage. With heat inactivated GPS, 14 out of the 20 eggs (70.0%) became blastocysts compared to 18 out of the 22 (81.8%) eggs cultured without GPS. These results indicate that a heat-labile component of GPS (perhaps the complement component) is responsible for egg death.

The presence of immune serum and Q serum does not significantly affect the development of blastocysts compared to that obtained in

TABLE 5.1

Invitro culture of C57BL and CBA F_1 hybrid eggs in immune serum and GPS.

			Antiserum titre	Eggs No.	Morula No.	Blastocysts	
						No.	%
25% GPS	Zona Intact	CBA antiserum	$4,096^{-1}$	4	0	0	0
		Peromyscus antiserum	$32,768^{-1}$	3	0	0	0
	Zona Free	CBA antiserum	$4,096^{-1}$	4	0	0	0
		Peromyscus antiserum	$32,768^{-1}$	4	0	0	0
10% GPS	Zona Intact	CBA antiserum	512^{-1}	8	7	1	12.5
		Peromyscus antiserum	-	8	8	2	25.0
	Zona Free	CBA antiserum	512^{-1}	8	0	0	0
		Peromyscus antiserum	-	8	0	0	0
Anti-serum only	Zona Intact	CBA antiserum	256^{-1}	6	6	5	83.3
		Peromyscus antiserum	$32,768^{-1}$	7	7	0	0
	Zona Free	CBA antiserum	256^{-1}	7	6	1	14.3
		Peromyscus antiserum	$32,768^{-1}$	6	6	0	0
Culture Medium only	Zona Intact			4	4	4	100
	Zona Free			4	4	3	75.0

Table 5.2

In vitro culture of zona pellucida free Q strain eggs in 10% GPS and mouse serum

Culture Medium	Eggs No.	Morula No.	Blast. No. (%)	Trophoblastic outgrowth		
				No.	% of Total Eggs	% of Blast.
Culture Medium alone	23	19	16 (69.6)	11	(47.8)	68.8
Inactivated immune serum and GPS	13	12	4 (30.8)	3	(23.1)	75.0
Inactivated immune serum & inactivated GPS	9	9	6 (66.7)	3	(33.3)	50.0
Immune serum and GPS	6	3	0 (0)	0	(0)	-
Immune serum & inactivated GPS	11	9	8 (72.7)	5	(45.4)	62.5
Immune serum only	5	5	3 (60.0)		NT	NT
GPS only	19	19	6 (31.6)		NT	NT
Inactivated immune serum	7	6	6 (85.7)	3	(42.9)	50.0
Inactivated Q serum and GPS	16	16	4 (25.0)	1	(6.3)	25.0
Inactivated Q serum	10	10	9 (90.0)	3	(30.0)	33.3

NT - Not tested

culture medium only. Heat inactivation of mouse serum seems to give eggs a particularly good chance of becoming blastocysts (with inactivated immune serum, 85.7% blastocysts, with Q serum, 90.0% blastocysts).

The presence of GPS does not appear to affect the formation of trophoblastic outgrowths once eggs have managed to develop to the blastocyst stage.

There was no improvement in the percentage of eggs developing to the blastocyst stage using a freeze-dried preparation of GPS. See Table 5.3.

Fresh GPS was found to be as toxic to zona-pellucida-free eggs at a 10% concentration as both the commercial preparations. When the concentration was decreased to 1%, 80% of eggs developed to the blastocyst stage. Results are given in Table 5.4.

Experiment III

The results of development of CBA eggs cultured in antiserum and control non-immune serum are given in Table 5.5. Details are given in Appendix 5.1. A selection of eggs was sacrificed during culture to be photographed at various stages of development. Plates 1-5 show examples of the egg stages recorded and typical examples of normal and abnormal blastocysts. Blastocyst formation was significantly lower ($p = 0.002$) in the antiserum group when paired observations were compared by culture days and analysed by the Binomial test. Subsequent development of embryos to trophoblastic outgrowth was also significantly lower in the presence of antiserum whether as a proportion of the total eggs set up in culture ($p = 0.011$) or of

TABLE 5.3

Invitro culture of Q strain eggs with 10% freeze dried GPS and 10% Q mouse serum.

Medium	Eggs No.	Morula No.	Blastocysts No.	Blastocysts %
Culture Medium	6	5	4	66.6
Mouse serum	10	7	5	50.0
GPS	10	6	2	20.0
Inactivated serum & GPS	13	10	4	30.8
Inactivated serum and inactivated GPS	12	10	5	41.7

TABLE 5.4

Invitro culture of Q strain eggs with 10% or 1% fresh GPS.

Medium	Eggs No.	Morula No.	Blastocysts No.	Blastocysts %
Culture Medium	10	9	5	50.0
10% GPS	17	10	5	29.4
1% GPS	10	9	8	80.0

TABLE 5.5*

Invitro culture of inbred CBA eggs in 10% C57BL anti-CBA serum or 10% C57BL serum.

GROUP	Eggs No.	Morula		Blastocysts		Trophoblastic Outgrowth		
		No.	%	No.	%	No.	% of Total eggs	% of blast.
C57BL anti-CBA serum	70	67	95.7	33	47.1	15	21.4	45.5
C57BL serum	65	61	93.8	48	73.8	36	55.4	75.0

TABLE 5.6*

Normal blastocyst development of CBA eggs in 10% C57BL anti-CBA serum or 10% C57BL serum.

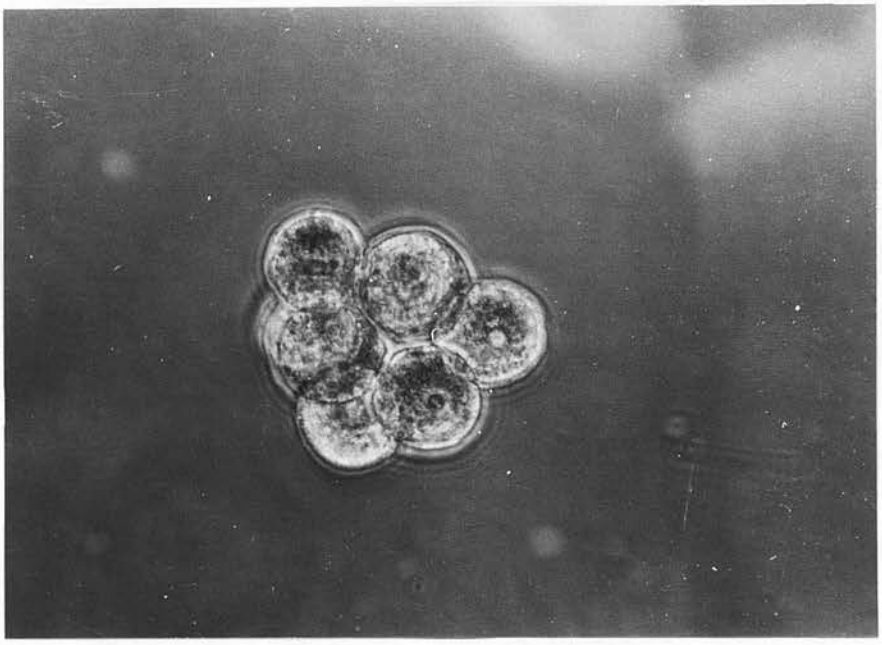
GROUP	Blastocyst No.	Normal No.	% Normal Blastocysts
C57BL anti-CBA serum	33	14	42.4
C57BL serum	48	40	83.3

* these figures represent the sums of daily series of experiments for individual figures. See Appendix 5.1.

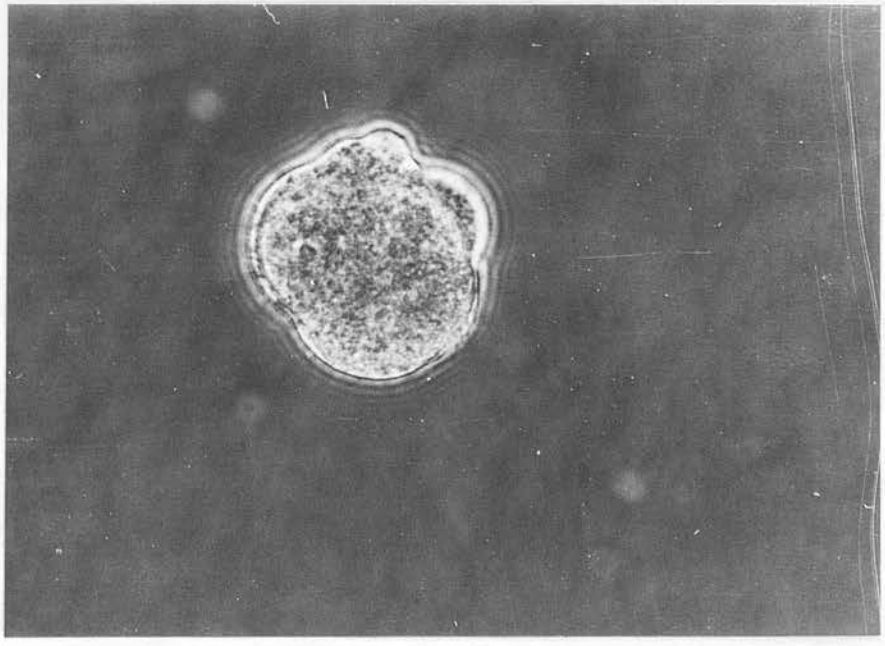
Plates of oviducal eggs to show the stages of development recorded.

Magnification x 400

Plate I

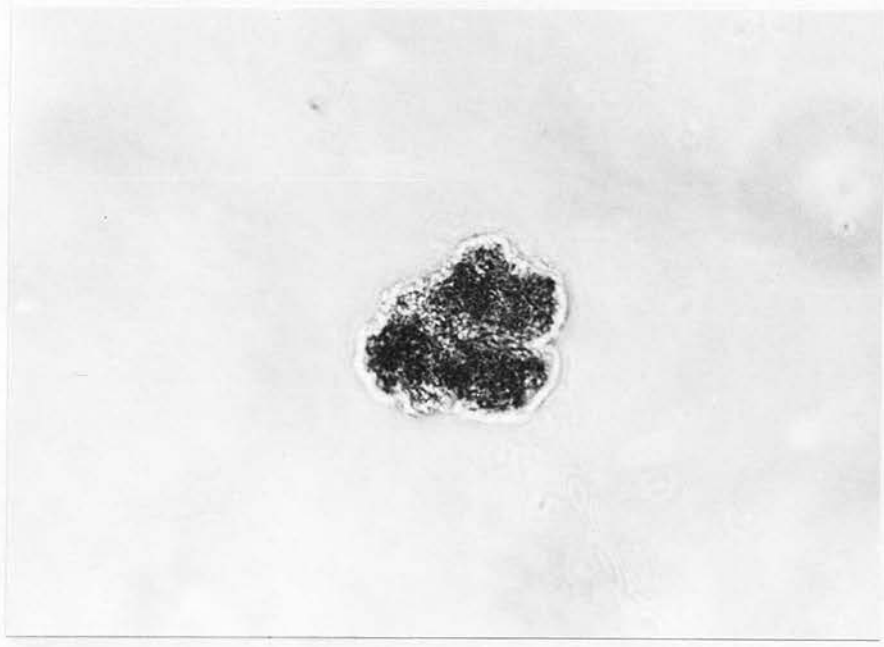


(a) Eight-cell egg with zona pellucida removed ready for culture.

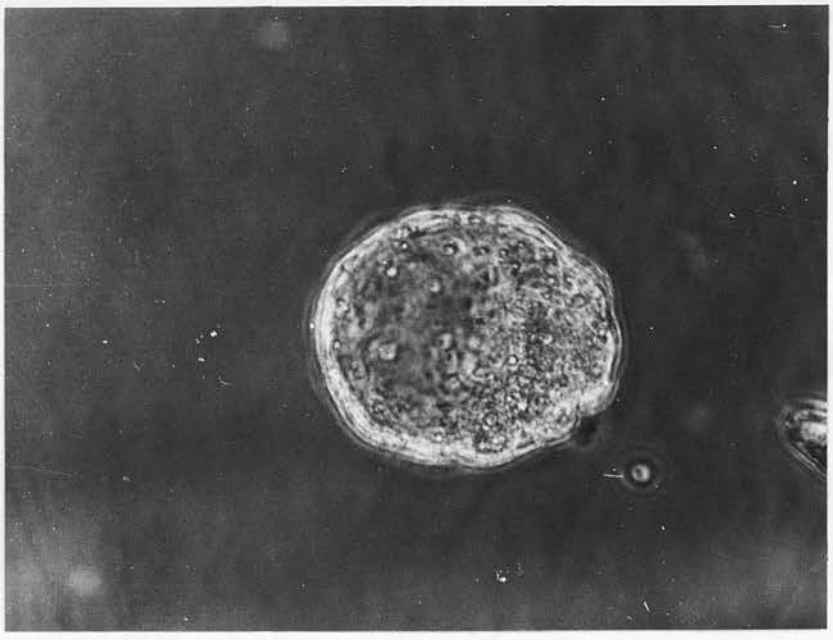


(b) Morula stage after 24 hours in culture.

Plate 2

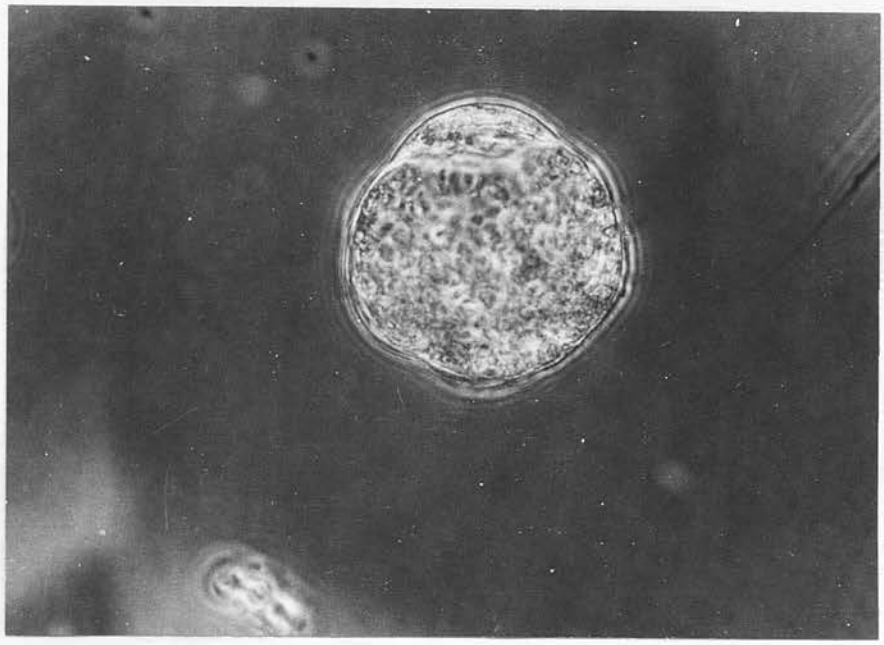


(a) A dead egg - 24 hours in culture.

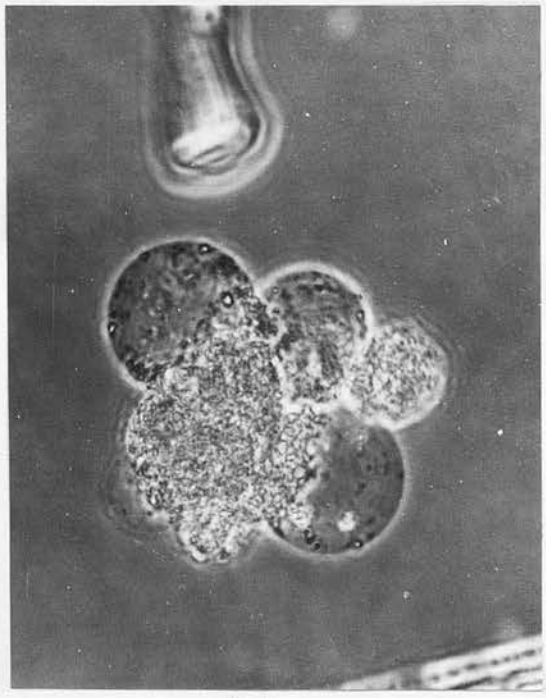


(b) Cavitation - 42 hours in culture.
The cavitation chamber is forming on the left-hand side of the egg.

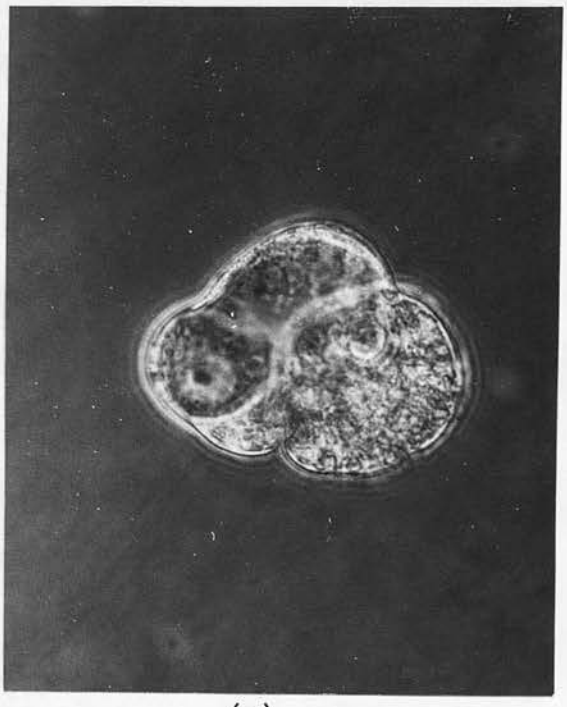
Plate 3



(a) Normal blastocyst with single cavity - 48 hours in culture.



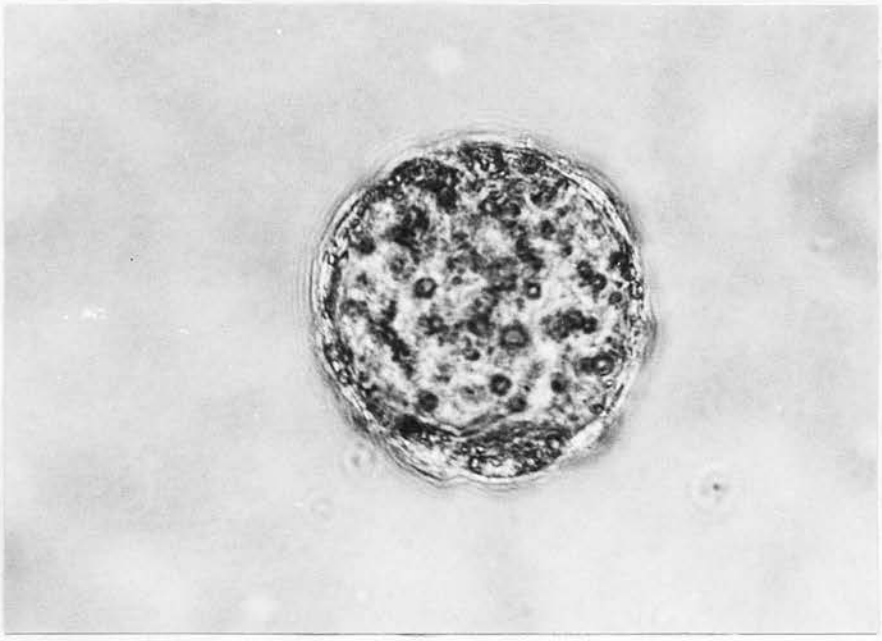
(b)



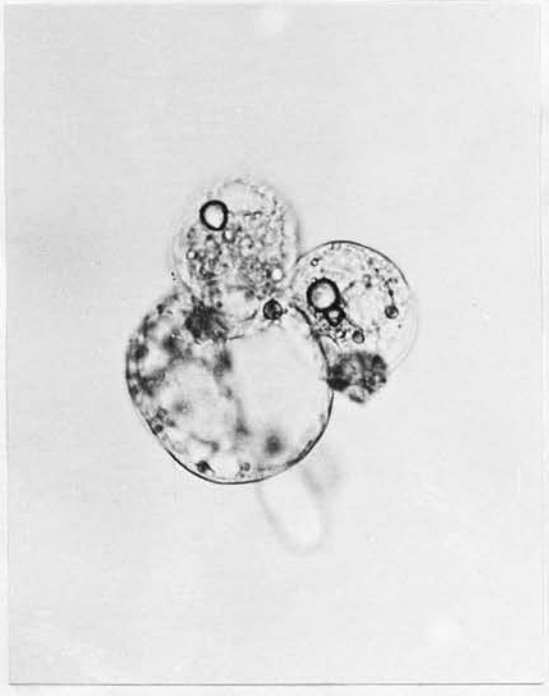
(c)

Abnormal blastocysts. (b) extrusion of dead cells on surface and several separate cavitations - 48 hours in culture. (c) no dead cells but three separate cavitations - 54 hours in culture.

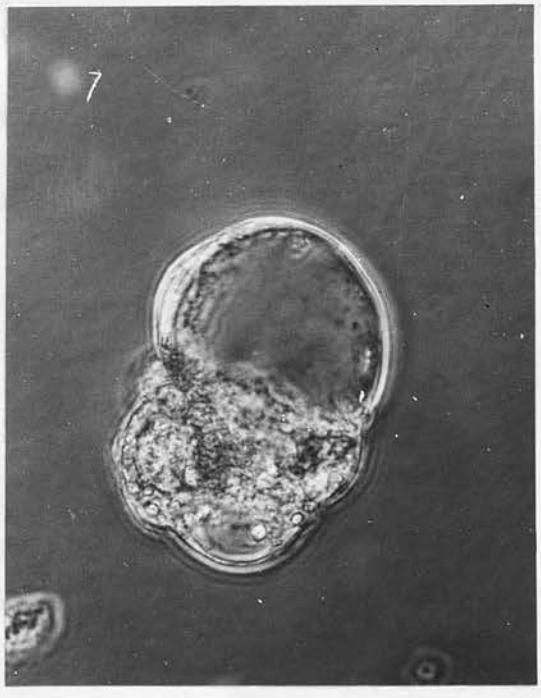
Plate 4



(a) Normal blastocyst - 58 hours in culture.



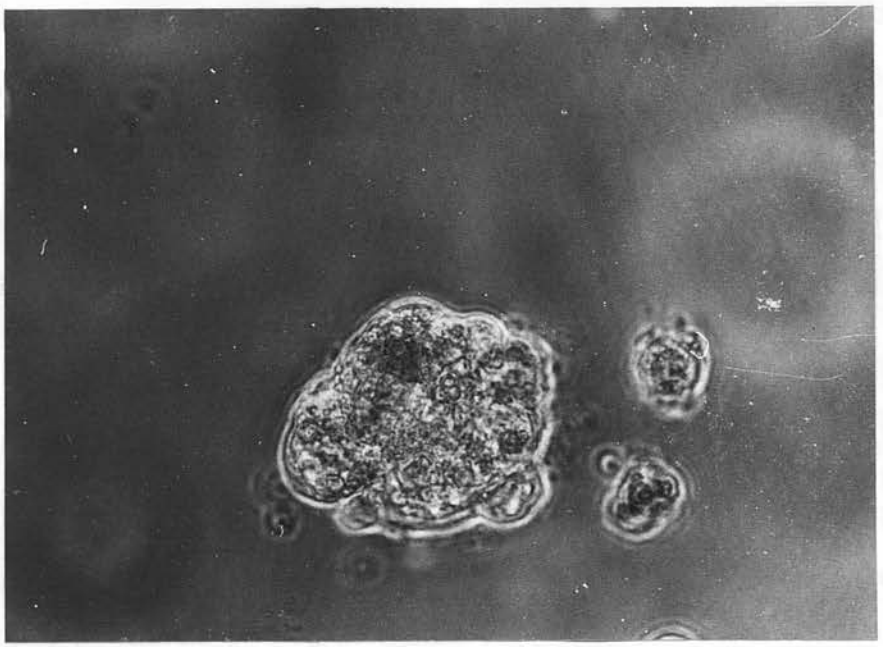
(b)



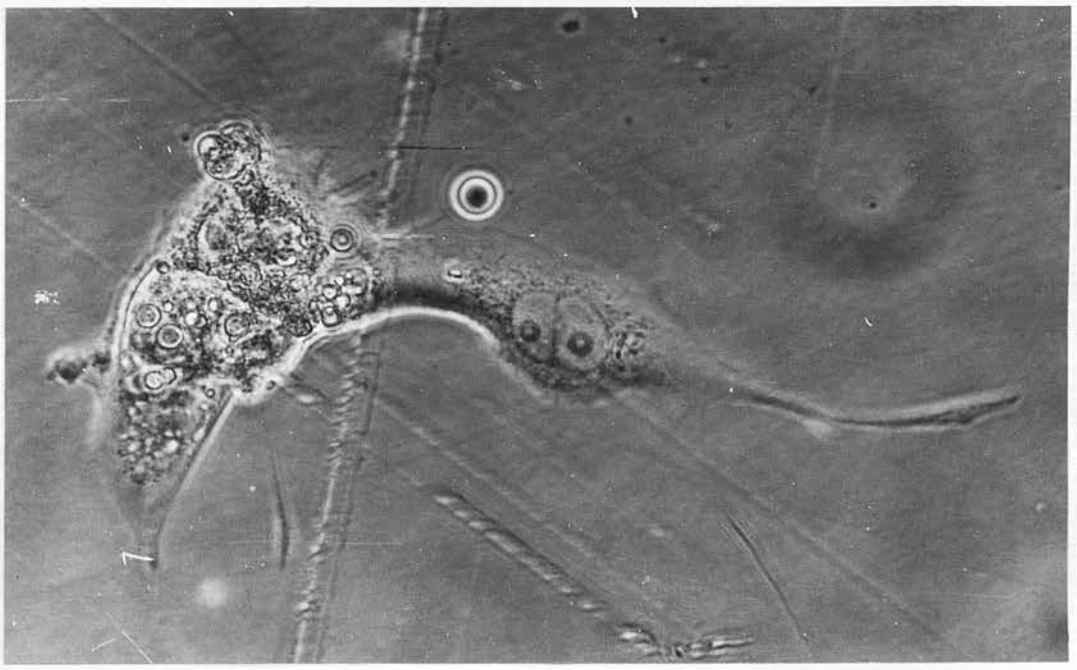
(c)

Abnormal blastocysts. (b) extrusion of two blebs which have cavitated separately. (c) only small % of cells have contributed to the blastocyst - 58 hours in culture.

Plate 5



(a) Blastocyst attachment. Blastocyst has collapsed onto surface of petri dish and is firmly attached - 72 hours in culture.



(b) Trophoblastic outgrowth. Cells have spread in a thin film over petri dish surface - 96 hours in culture.

the number of blastocysts ($p = 0.035$). The weighted regression of percentage of blastocysts on serum antibody titre was negative and statistically significant ($F = 6.046$, d.f. 1 and 14, $p < 0.05$), showing that the number of eggs developing into blastocysts falls as the titre of antibody increases (See Figure 1). Data from CBA egg culture in Experiment IV were included in this analysis.

Table 5.6 shows that there is a significant reduction in the proportion of normal blastocysts when eggs were grown in antiserum ($p = 0.035$). However, regression analysis of the percentage normal blastocysts on titre was not significant ($F = 2.54$, d.f. 1 and 12, $p < 0.20$) although in the expected direction.

There was no correlation between the success of a blastocyst in forming a trophoblastic outgrowth and the antibody titre of the serum in which it was cultured.

It was was noted in the course of recording blastocyst development that CBA eggs in the immune serum took longer to become blastocysts than eggs in the control serum. The time of cavitation of eggs was therefore calculated. Eggs were divided into the percentage of eggs which had cavitated by forty eight hours in culture and the percentage which cavitated after forty eight hours (see Appendix 5.1 for details). Table 5.7 shows that whereas 92.5% of eggs in the control serum had cavitated by forty eight hours, only 66.7% of those in antiserum had done so. Eggs grown in antiserum showed a delay of eight hours in cavitation compared to eggs grown in control serum. However, when only eggs were compared which later formed normal blastocysts, this eight hour difference was reduced to one hour difference. (See Figure 2). Data from CBA egg culture in Experiment IV

TABLE 5.7

The time of cavitation of CBA eggs grown in 10% antiserum or control serum.

GROUP	CAVITATION by 48 hours				CAVITATION later than 48 hours			
	Total Eggs		Normal Blast.		Total Eggs		Normal Blast.	
	No.	%	No.	%	No.	%	No.	%
C57BL anti-CBA serum	22	66.7	12	54.5	11	33.3	2	18.2
C57BL serum	37	92.5	31	83.8	3	7.5	2	66.7

Figure 1. The effect of antibody titre on % CBA blastocysts

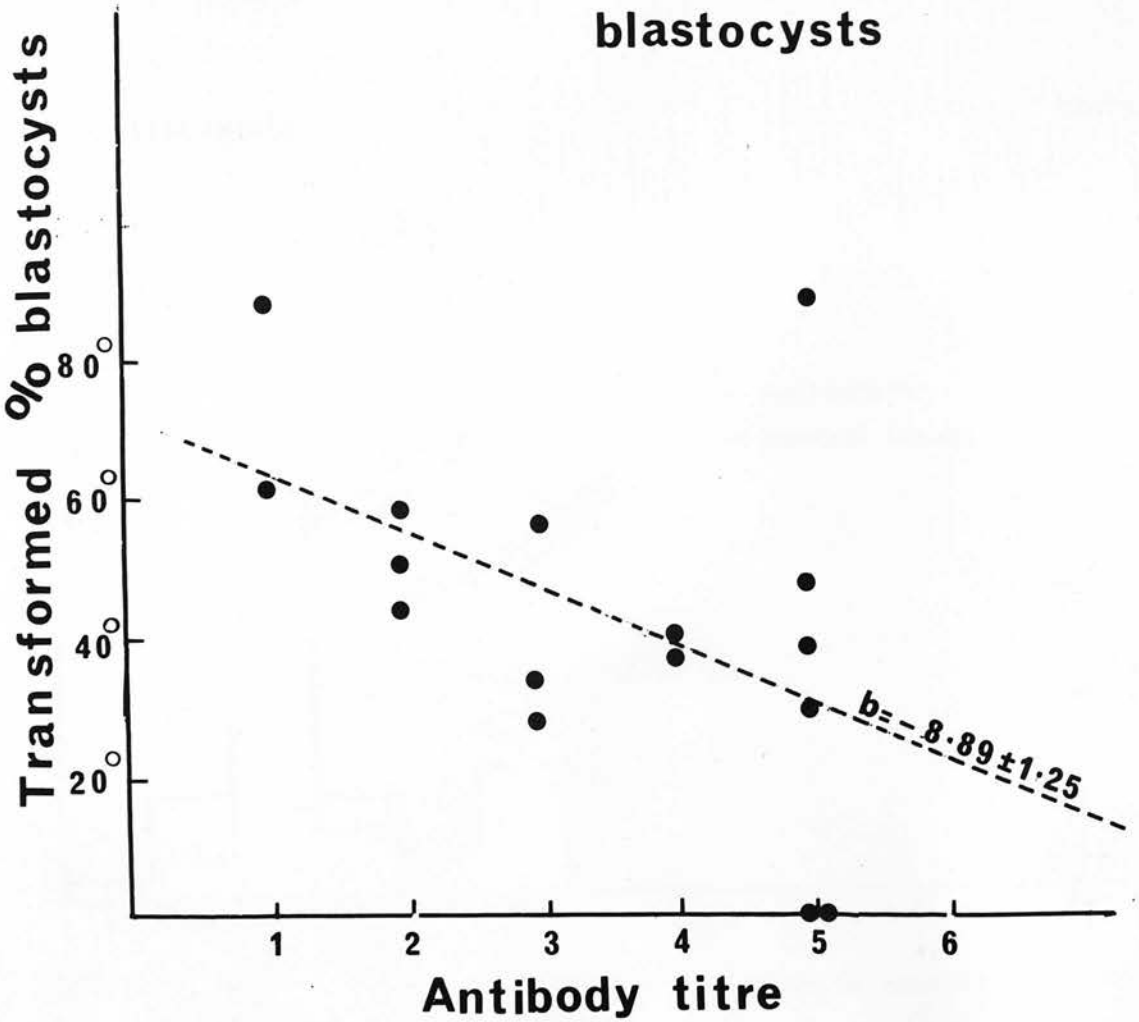
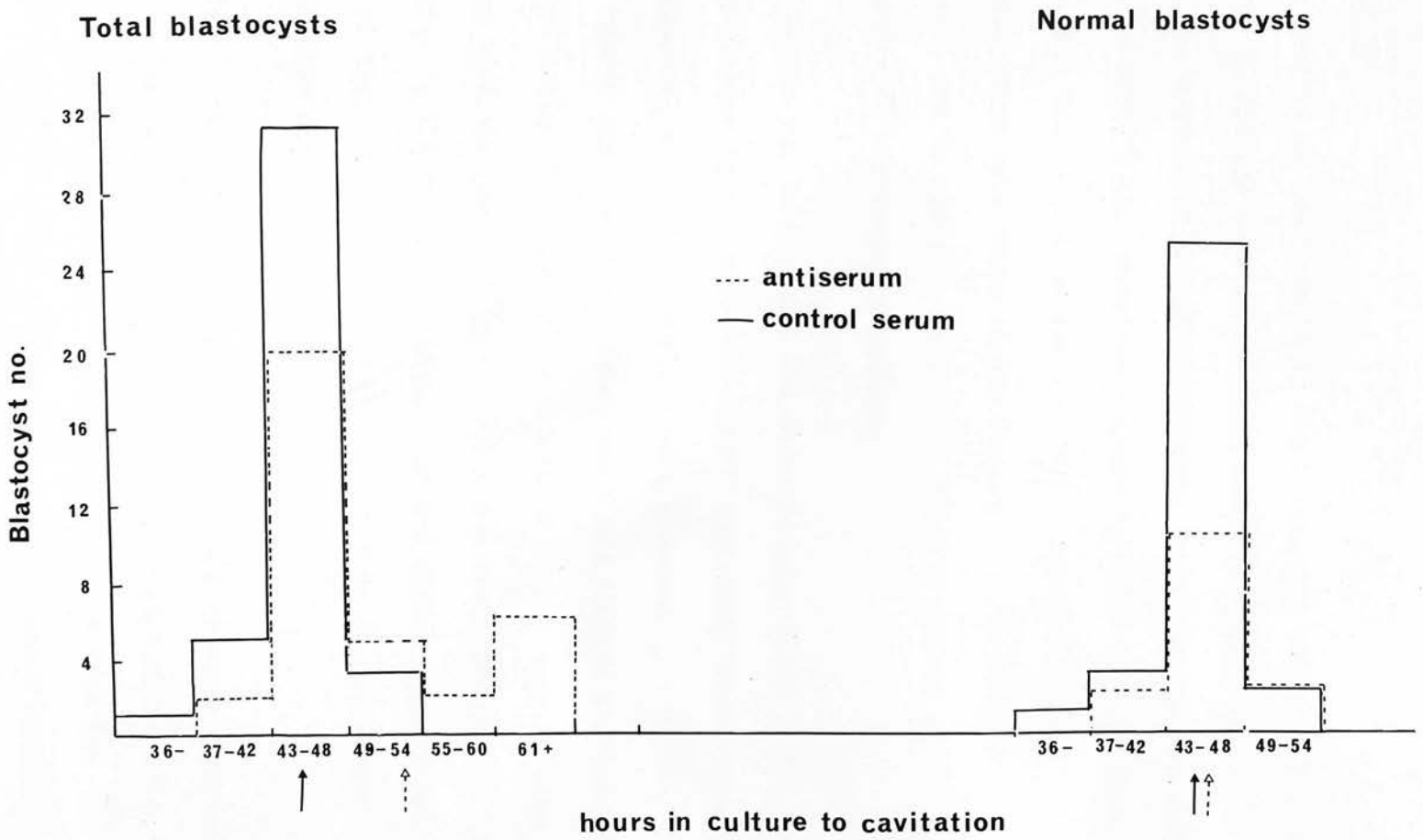


Figure 2. Cavitation time of CBA eggs in antiserum and control serum



were included in this analysis. From this experiment it is not possible to distinguish whether eggs which are destined to form abnormal blastocysts take longer to cavitate than eggs which will form normally or eggs which are delayed and cavitate late are more likely to be abnormal.

Experiment IV

The results of development to the blastocyst stage of C57 BL x CBA F_1, C57 BL and CBA eggs in anti-CBA serum are given in Tables 5.8 and 5.9. The numbers of normal blastocysts formed in the two series are given in Table 5.10. Data details can be found in Appendices 5.2 and 5.2a. The results of the two series did not differ significantly from each other in any respect and for the purpose of analysis they can be pooled. Table 5.11 gives a summary of the pooled results of blastocyst formation.

CBA blastocyst development was significantly lower than that in the C57 BL eggs ($p < 0.025$, one-tailed) and among those blastocysts that did develop, a higher proportion were abnormal ($p < 0.001$, one-tailed). Hybrid egg blastocyst development was similar to that of C57 BL eggs, a significantly larger number of hybrid eggs forming blastocysts than the CBA eggs ($p < 0.05$) and a lower proportion forming abnormalities ($p < 0.002$). Hybrid eggs do not differ significantly from C57 BL eggs in blastocyst development but do in the proportion of abnormal blastocysts ($p < 0.05$).

There was no significant difference between the three groups in the percentage of blastocysts which formed trophoblastic outgrowths. Blastocyst abnormality did not appear to affect the capacity of an egg to attach and outgrow. Information on attachment and trophoblastic outgrowth was limited in Series I because of a

TABLE 5.8*

In vitro culture of C57 BL x CBA, C57 BL and CBA eggs in 10% C57 BL anti-CBA serum and 1% GPS - Series I.

GROUP	Eggs No.	Morula		Blastocysts		Trophoblastic Outgrowth			
						No.		%	
		No.	%	No.	%	of egg Total	of Blast.	of egg Total	of Blast.
C57 BL x CBA	75	64	85.3	64	85.3	17/30	17/26	56.7	65.4
C57 BL	65	61	93.8	49	75.4	14/30	14/27	46.7	51.9
CBA	80	64	80.0	51	63.8	19/50	19/25	38.0	76.0

TABLE 5.9*

In vitro culture of C57 BL x CBA, C57 BL and CBA eggs in 10% C57 BL anti-CBA serum and 10% GPS - Series II.

GROUP	Eggs No.	Morula		Blastocysts		Trophoblastic Outgrowth		
							%	
		No.	%	No.	%	No.	of egg Total	of Blast.
C57 BL x CBA	36	31	86.1	26	72.2	22	61.1	84.6
C57 BL	34	34	100.0	31	91.2	25	73.5	80.6
CBA	49	47	95.9	27	55.1	20	40.8	74.1

* These figures represent the sums of daily series of experiments.

For individual figures see Appendices 5.2 and 5.2a.

TABLE 5.10

Normal blastocyst development of C57BL x CBA F_1 hybrid, C57BL and CBA eggs in C57BL anti-CBA serum and 1% or 10% GPS.

	GROUP	NO. BLAST.	NO. NORMAL	% NORMAL
Series I 10% antiserum 1% GPS	C57BL x CBA	59	53	89.8
	C57BL	33	29	87.9
	CBA	21	4	19.0
Series II 10% antiserum 10% GPS	C57 x CBA	26	20	76.9
	C57BL	31	31	100.0
	CBA	27	14	51.9

Table 5.11

Summary of blastocyst formation

Eggs	% blastocyst development	% blastocyst normality
Hybrid	81.1	85.0
C57 BL	80.8	93.7
CBA	60.5	37.5

technical difficulty. A 1% solution of calf serum is normally added to culture drops after forty eight hours in order to promote egg attachment. However, on the several occasions it was added, all further development was arrested. When this addition was not made, attachment and outgrowth occurred spontaneously. Information on attachment and outgrowth was made only from cultures in which calf serum was not added. It is likely that its presence, in addition to the mouse and guinea pig serum already present in the medium raised the concentration of serum too high for egg viability.

The regressions on antibody titre of the percentage of eggs developing into blastocysts and the percentage showing normal development were not statistically significant in any of the three groups.

Discussion

a) The toxicity of guinea pig serum to developing eggs

Previous studies involving the use of GPS in egg culture experiments have taken little or no account of the problem of its toxicity to 'zona free' eggs. Heyner et al. (1969) examined the influence of immune serum and complement on the development of tubal mouse eggs, but did not state the source of GPS used. Personal communication to Dr. A. McLaren has established that fresh GPS was used. They claim that development was unaffected by the presence of GPS. However, inspection of their results shows that eggs cultured in 25% GPS formed fewer blastocyst aggregates when compared with eggs cultured without GPS. Heyner et al. lowered the concentration of GPS from 25% in the first experiment to 10% in later experiments and compared the results of egg development. Since the presence of 10% GPS in their cultures did not appear to cause a deficient formation of blastocyst aggregates, it does not seem advisable to compare egg development in cultures containing different amounts of GPS.

James (1969) using Bacto complement (Difco) in a 10% solution reports toxic effects on 'zona free' blastocyst formation.

Thus, in the experiments of Heyner et al., 10% GPS gave no evidence of toxic effects, in James' experiments it showed some degree of toxicity, and in my cultures it showed considerable toxicity. These facts suggest that much may depend on the particular batch of GPS used. This suggestion is supported by the lower toxicity of the GPS used in Experiments III and IV (see next sections), which allowed the use of 10% GPS despite the discouraging results of Experiments I and II.

The presence of mouse serum or antiserum in the culture medium does not significantly affect blastocyst formation, whether heat inactivated or not. However, the percentage of blastocysts was non-significantly higher after inactivation, suggesting that mouse complement may have had some slight toxic effect.

b) The effect of anti-CBA serum on CBA egg development

The effect of anti-CBA serum in reducing the capacity of CBA eggs to form blastocysts appears to be due specifically to the presence of anti-CBA antibodies. This observation is strengthened by the finding that the percentage of eggs successfully developing into blastocysts significantly falls as the titre of serum antibody to which they are subjected increases. This result is in agreement with the findings of Heyner et al. (1969) that blastocyst formation is reduced when 'zona free' C3H eggs are cultured with DBA/2 anti-C3H serum.

Several other parameters of CBA egg development are also affected by the presence of antiserum. A significantly higher proportion of eggs that reached the blastocyst stage are abnormal when development occurred in antiserum, although the proportion of abnormal blastocysts was not dependent on antibody titre. Heyner et al. (1969) do not report a similar occurrence of abnormal blastocysts but it is doubtful if their method of culture would allow a distinction to be made between normal and abnormal blastocysts. They did not culture eggs singly in drops but put eggs undergoing one treatment in one drop. Eggs which have had the zona pellucida removed adhere in a culture drop to form a variable number of large aggregate

blastocysts rather than forming one blastocyst for each egg. Under these conditions abnormality of individual blastocysts is likely to be obscured.

In addition to the effect on blastocyst development, anti-CBA antibodies impair subsequent development of trophoblastic outgrowth, which also appears independent of antibody titre. This is consistent with the results of James (1969) in an in vivo study of trophoblastic development. He found that C57 BL blastocysts cultured in the presence of C3H anti-C57 BL serum showed lower trophoblast viability when transplanted to C3H kidney capsules than eggs which had been cultured in non-immune serum.

The adverse effects of antiserum result in the formation of fewer blastocysts, increased abnormality and retarded cavitation. These results indicate the presence of transplantation antigens on eggs prior to blastocyst formation. The retarded appearance of a cavitation chamber is strongly associated with the formation of an abnormal blastocyst. It is not known whether the formation of an abnormal blastocyst is directly due to the action of antiserum or whether it is a secondary effect resulting from retarded development. The presence of antiserum may retard development by interfering with cell division, leading to both late caviation and to abnormality. This could be tested by comparing cell counts of eggs culture in immune serum and non-immune serum.

c) The effects of anti-CBA serum on C57 BL x CBA F_1 hybrid egg development

The results of growing CBA eggs in anti-CBA serum agree with the results in the last experiment.

The results from culture of hybrid eggs show their viability in antisera is much nearer that of C57 BL eggs than that of CBA eggs. However, although C57 BL and hybrid egg development was similar in respect to blastocyst formation, hybrid eggs did form larger numbers of abnormal blastocysts. In this respect they are less viable than C57 BL eggs. This suggests that paternal antigens can be detected in the hybrid blastocyst but their expression is proportionally much weaker than would be expected from a consideration of gene dosage.

It might be argued that CBA eggs are inheritantly less viable than C57 BL under the conditions of culture and that the intermediacy of hybrids is a consequence of gene dosage. This possibility is excluded by the results of Experiment III. CBA egg viability in the absence of antiserum in Experiment III is very similar to the viability of C57 BL eggs in the present experiment.

It would be interesting to test directly for the low density of paternal antigens on hybrid eggs by using two types of antisera on fertilized eggs from reciprocal crosses. If it is a genuine phenomenon, weak expression of paternal antigens may contribute to protecting the early embryo against the adverse effects of maternal immunity.

Chapter VI

The effects of active and passive preimmunization on the decidual cell reaction of pregnancy

The effects of active and passive preimmunization on the decidual cell reaction of pregnancy

Introduction

While the placenta may form a barrier that prevents the immunological rejection of the foetus, some other mechanism must be involved during implantation and before the formation of the definitive placenta. The actual process of implantation involves a complex series of interactions between endometrium and blastocyst, the nature and control of which are poorly understood. This phase of the reproductive cycle is, nevertheless, of great importance, for it is during this period that the highest mortality of embryos occurs (Hertig, Rock, Adams and Menkin, 1957; Brambell, 1948; Adams, 1955).

There are a number of observations which have led to the suggestion that immunological reaction could occur between mother and foetus at the time of implantation. In the rabbit, accumulations of lymphocytes occur in the sub-epithelial space at the site of implantation (Potts, 1965), and antigens present in the blastocoelic fluid have been recovered from the uterine stroma and lumen (Beier, 1968). In the rat, uterine cells transfer substances into the sub-epithelial space at the site of implantation (Vokaer, 1952). These observations indicate that the passage of substance between blastocyst and uterus is possibly quite frequent.

One of the early signs of implantation in many mammals is the development of decidual tissue in the uterine stroma in the region of the blastocyst. When pre-trophoblastic stages of development are transplanted to ectopic sites they are rejected if the host has been

sensitized to the transplantation antigens of the donor. When transferred to the uteri of similarly prepared hosts, in the presence of decidual tissue, rejection does not occur (Kirby et al., 1966). This observation led to the suggestion that decidual tissue may provide protection from immunological consequences of maternal/foetal incompatibility during this early stage of development. The nature of the protection is unclear, as decidual tissue is well supplied with blood and lymphatic vessels. The presence of tight junctions between cells in decidual tissue, reported by Finn and Lawn (1967), may be relevant in limiting cellular contact and passage of cells between the mother and conceptus at this stage.

There is only meagre evidence that immunological interactions around the time of implantation significantly affect development. From measurements of the amount of decidual tissue in mice on the 7th day of pregnancy Hetherington (1970) has postulated that during the pre-implantation phase of pregnancy an immunological reaction occurs which affects either the blastocyst's ability to induce the decidual cell reaction or the mother's ability to respond. Variations in the amount of decidual tissue were not due to differences in the rate of development of the pre-implantation blastocyst, which is known to depend on both the blastocyst genotype and the maternal environment (Whitten and Dagg, 1961; Gates, Doyle and Noyes, 1961; McLaren, 1968).

This observation induced an investigation into the effects on the decidual cell reaction of maternal preimmunization to paternal antigens.

Studies were made on both the effects of active immunization (Experiments I & II) and the effects of passively transferred isoantisera (Experiments III & IV). Experiments I and II were designed to investigate

the decidual response in females actively immunized to the antigens of the mating male. Experiment I compared the effects on decidual weights of allogeneic and xenogeneic immunization. Experiment II, in addition, examined the results of using different methods of allogeneic immunization. Experiments III and IV used passive immunization to determine the effects of humoral factors in the absence of cellular immunity.

Experiment I and Experiment IV were carried out in collaboration with Dr. C. Hetherington. A publication reporting the results of Experiment I is enclosed, (Appendix 6.4).

A. The effects of active immunization - Experiments I and II

1. General Materials and Methods

Female C57 BL mice aged between eight and twelve weeks were distributed at random into groups. Mice were either immunized allogeneically to CBA male tissue, xenogeneically to Peromyscus antigens or left as unimmunized controls. In Experiment I one group of mice was immunized allogeneically, while in Experiment II three groups were immunized allogeneically using different methods of preimmunization. The methods of skin grafting and spleen cell injection were carried out as described in previous chapters. All treatments were given at 14 day intervals. Six days after the last injection of spleen cells all the mice were mated to CBA males. On the seventh day of pregnancy (vaginal plug = 1st day) the mice were killed and dissection was carried out in P.B.S. to remove the decidual tissue from the uterus. No attempt was made to remove the tissue of the conceptus. The decidua from each uterus were blotted lightly, pooled, and weighed to the nearest 0.1 mg. The mean decidual weight per implantation site was calculated for each female. At autopsy the females were bled to determine the levels of serum antibody against CBA and Peromyscus red blood cells. Haemagglutination was carried out using the human serum dextran method of Gorer and Mikulska (1954). In Experiment I, lumbar lymph nodes and spleens were dissected from each female at autopsy and were weighed.

The mean decidual weights of the groups were compared using the analysis of variance. Individual group means were compared using the Student's t test. The regression of decidual weight on antibody titre was also examined.

In Experiment II, Student's t test was used to compare the titres obtained by different methods of immunization.

2. Experiment I

Allogeneic and xenogeneic immunization was carried out with two full thickness skin grafts and three injections of spleen cells. Autopsy was carried out on the morning of the 7th day of pregnancy.

Results

The results are recorded in Table 6.1, and more detailed information is given in Appendix 6.1. The mean decidual weight for females immunized to CBA antigens was significantly lower than the mean weight of either the untreated controls, or the group immunized to Peromyscus antigens (for heterogeneity between groups; $F = 10.11$; d.f. 47 & 2; $p < 0.001$). There was no significant difference between the untreated controls and the xenogeneically immunized group. The effect of immunization with cells of the paternal strain is therefore specific, and neither the result of a general non-specific heightening of immunity, nor of stress experience during immunization.

Sera from allogeneically immunized females produced haemagglutination titres ranging from 1/8 to 1/4,096. Sera from xenogeneically immunized mice gave high titres (1/1,024 to 1/32,768) when tested against Peromyscus red blood cells. Sera from control animals showed no activity with either antigen.

Mice immunized both allogeneically and xenogeneically had significantly larger lymph nodes ($F = 15.00$; d.f. 46 and 2; $p < 0.001$), and spleens ($F = 4.01$; d.f. 47 and 2; $p < 0.05$) than controls.

The mean implantation number in the allogeneically immunized mice was lower than in the other groups but the reduction was not significant.

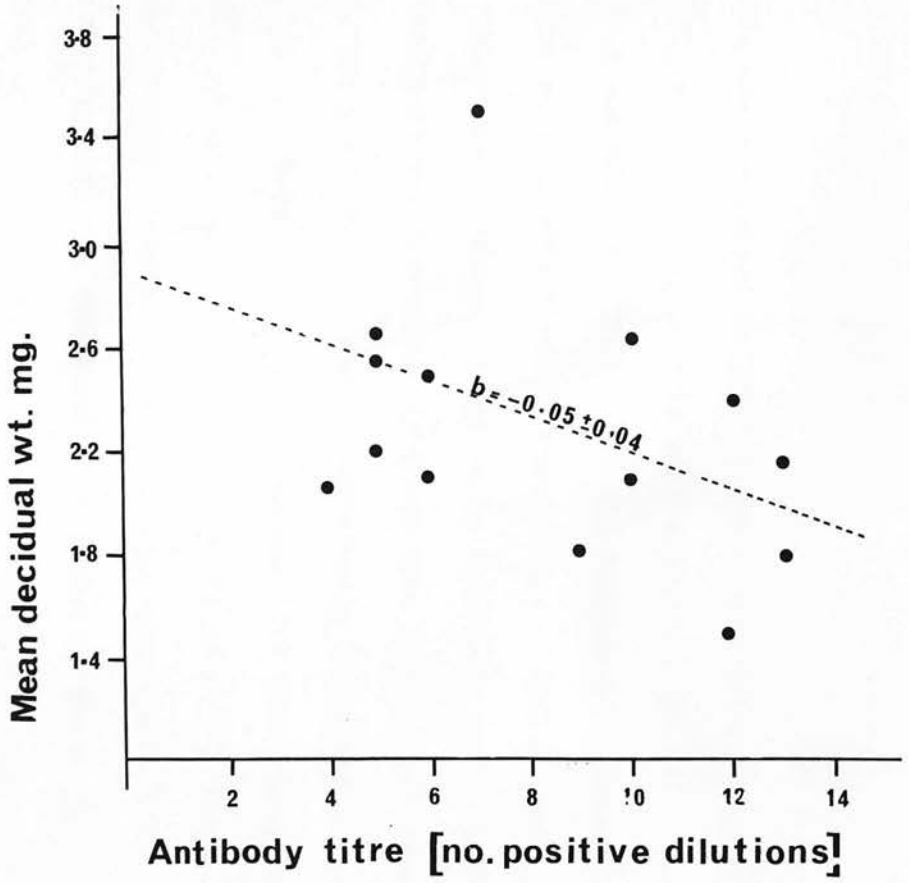
The slope of the regression of decidual weight on antibody titre was negative but was not significant ($b = -0.05 \pm 0.04$; $p > 0.05$: see Figure 1).

Table 6.1

Decidual weights in allogeneically immunized, xenogeneically immunized and non-immunized C57 BL mice - Experiment I

Group	No. of Females	Mean Implantation no. ± s.e.	Mean decidual wt. mg ± s.e.	Mean lumbar node wt. mg ± s.e.	Spleen wt. mg ± s.e.	Mean serum antibody titre (no. +ve dilutions)
A Allogeneically Immunized	14	7.5 ± 0.6	2.29 ± 0.13	6.31 ± 0.4	122.1 ± 4.1	256^{-1} (8)
B Xenogeneically Immunized	17	8.2 ± 0.4	3.25 ± 0.20	7.31 ± 0.6	126.2 ± 4.2	$16,384^{-1}$ (14)
C Non-immunized Controls	17	8.6 ± 0.3	3.27 ± 0.15	3.97 ± 0.4	119.9 ± 2.9	-

Figure 1. The effect of antibody titre on decidual weight – Expt. I



3. Experiment II

Three groups of mice were immunized allogeneically to paternal antigens. The first group (A) received, as in the last experiment, two skin grafts and three spleen cell injections. The second (B) received two skin grafts and the third (C) was immunized with three injections of spleen cells. It was hoped that the different methods of immunization would throw light on the type of immunity responsible for decreased decidual size. Immunization with skin grafts is known to lead to a high level of cellular immunity, whereas injections of spleen cells lead to a relative predominance of humoural immunity. Xenogeneically immunized mice (Group D) received two full thickness skin grafts and three spleen cell injections from Peromyscus. Autopsy was carried out in the afternoon of the seventh day of pregnancy.

Results

The results are summarized in Table 6.2 and data details are given in Appendix 6.2. Mean decidual weights were significantly heterogeneous ($F = 3.65$, d.f. 4 and 62; $p = 0.001$). Not all methods of allogeneic immunization were equally effective in depressing decidual weight. The mean decidual weight from females immunized by both skin grafts and spleen cells (Group A) was significantly lower than the control group ($t = 3.29$; d.f. 22; $p < 0.01$) and the xenogeneically immunized group ($t = 2.3$; d.f. 25; $p < 0.05$). This result agrees with that found in the last experiment. Group B, immunized with spleen cells only, showed a significantly lower mean decidual weight than the controls ($t = 2.6$; d.f. 22; $p < 0.02$) but not the xenogeneically immunized group. Immunization with skin grafts (Group C) gave an intermediate mean decidual weight that did not differ significantly from any other group.

Table 6.2

Decidual weights in allogeneically immunized, xenogeneically immunized and non-immunized C57 BL mice - Experiment II

Group	No. of females	Mean implantation no. ± s.e.	Mean decidual wt. mg. ± s.e.	Mean serum antibody titre (no. +ve Dilutions)
A Allogeneically immunized with skin graft and spleen cells	13	9.1 ± 0.4	4.45 ± 0.16	256^{-1} (8)
B Allogeneically immunized with spleen cells only	15	8.4 ± 0.3	4.63 ± 0.15	16^{-1} (4)
C Allogeneically immunized with skin grafts only	14	9.1 ± 0.4	4.90 ± 0.15	32^{-1} (5)
D Xenogeneically immunized	14	9.0 ± 0.3	4.96 ± 0.14	N.T.
E Non-immunized controls	11	7.8 ± 0.4	5.28 ± 0.19	-

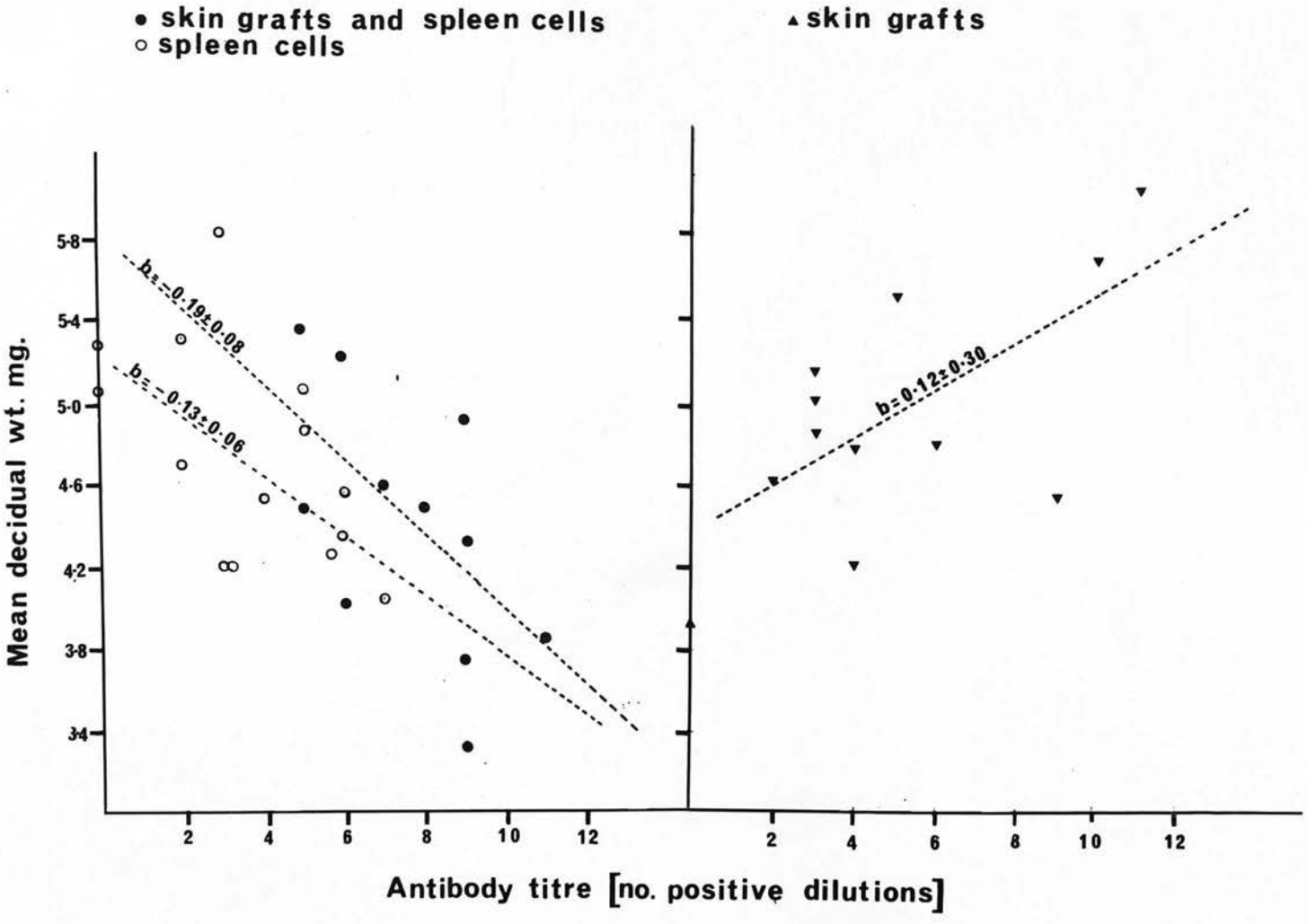
Sera from the three allogeneically immunized groups gave haemagglutination titres ranging from -ve to 1/2,048. Antibody titres of sera from Group A females were considerably higher than those of Group B or C. ($F = 7.81$; d.f. 2 and 34; $p < 0.01$). Titres from females immunized with both skin grafts and spleen cells were significantly higher than those from females immunized by spleen cells ($t = 4.95$; d.f. 23; $p < 0.001$) or skin grafts ($t = 2.3$; d.f. 21; $p < 0.05$). There was no significant difference in titres between Groups B and C.

It is apparent therefore, that immunization using skin grafts alone gives antibody titres similar to those obtained using spleen cells alone. However, whereas the immunity produced by spleen cells significantly reduces decidual weight, the immunity produced by skin grafts does not.

The regressions of decidual weight on antibody titre show that decidual weight is dependent on antibody titre in Groups A and B but not in Group C (immunized with skin grafts only; see Figure 2). The slopes of the three regressions are significantly heterogeneous ($F = 7.90$; d.f. 2 and 31; $p < 0.01$). There is a significant negative relation between decidual weight and antibody titre in Group A ($b = -0.19 \pm 0.08$; $p < 0.05$) and Group B ($b = -0.13 \pm 0.06$; $p < 0.05$). There was no such pattern in Group C where the regression was positive but not significant ($b = 0.12 \pm 0.31$; $p > 0.05$).

There is no difference between the slopes of Groups A and B ($b = -0.15 \pm 0.04$; $F = 0.29$) nor do their elevations differ ($F = 1.66$; d.f. 1 and 22; $p > 0.05$). Group C however, shows a slope significantly different from the other two groups (from A; $F = 10.9$; d.f. 1 and 19, $p < 0.01$: from B; $F = 9.96$, d.f. 1 and 22; $p < 0.01$).

Figure 2. The effect of antibody titre on decidual weight – Expt. II



There was no significant difference between the groups in mean numbers of implantations.

B. The effects of passive immunization - Experiments III and IV

1. Experiment III

a) Materials and Methods

The experiment was carried out in two series. In the first series C57 BL females, pregnant to CBA males, received immune or non immune anti-sera. The immune sera came from C57 BL female mice which had been immunized to CBA antigens with one skin graft and one injection of spleen cells. In the second stage, pregnant mice received either sera obtained by immunization with two skin grafts and three spleen call injections, or non immune sera.

The immune sera were collected by bleeding immunized mice eight days after their last spleen cell injection. The blood was pooled, kept at room temperature for two hours and left overnight at 4° C to clot. It was then centrifuged. The serum was removed and kept at -20° C until use. Haemagglutination titres were not measured. 0.05 ml of serum was injected intraperitoneally on the third and fifth days of pregnancy (first day = day of vaginal plug). The recovery of decidua was made on the afternoon of the 7th day of pregnancy. Statistical analysis was carried out as described above.

b) Results

In the first series, mice receiving immune serum showed no reduction of decidual weight. In the second series, however, the reduction was significant ($t = 3.39$; d.f. 6; $p < 0.02$; see Table 6.3 and Appendix 6.3 for detailed data). There was no significant effect on implantation numbers at either stage.

These results suggest that the effect of immune serum depends upon the strength of the immunity.

Serum from weakly immunized donors does not reduce decidual weights, but serum from strongly immunized donors seems to be effective.

Table 6.3

Decidual weights in passively immunized C57 BL mice - Experiment III

	Group	No. of females	Mean implantation no. ± s.e.	Mean decidual wt. mg ± s.e.
Series I	A Immune serum (1 s.g. and 1 s.c.)	4	7.0 ± 1.08	5.56 ± 0.16
	B Control serum	6	6.2 ± 0.98	5.54 ± 0.22
Series II	C Immune serum (2 s.g. and 3 s.c.)	4	8.5 ± 0.50	4.79 ± 0.23
	D Control serum	4	8.3 ± 0.45	5.66 ± 0.12

s.g. = skin grafts
s.c. = spleen cells

2. Experiment IV

a) Materials and Methods

This experiment was similar to the second series of Experiment III. It was carried out at the Clinical Research Centre of Northwick Park Hospital, and used their populations of mice. Immune serum was obtained from C57 BL female mice that had been immunized to CBA males by two full-thickness skin grafts, followed by three spleen cell injections. On the third day of pregnancy (first day = day of vaginal plug), C57 BL mice mated to CBA males received 0.5 ml of either immune or non-immune serum. On the 4th day the mice received a further 0.2 ml of serum. Mice were killed on the morning of the 7th day of pregnancy and the decidua were harvested as before.

b) Results

Mice receiving immune serum showed a significant reduction of decidual weight ($t = 2.42$; d.f. 16; $p < 0.05$). See Table 6.4 and Appendix 6.3 for details. There was no effect on the implantation number. This result confirms the earlier experiment.

Table 6.4

Decidual weights in passively immunized C57 BL mice - Experiment IV

Group	No. of females	Mean Implantation no. ± s.e.	Mean decidual wt. mg. ± s.e.
Immune serum (2 s.g. and 3 s.c.)	8	8.4 ± 0.6	3.08 ± 0.18
Control serum	10	7.6 ± 0.7	3.61 ± 0.14

s.g. = skin grafts
s.c. = spleen cells

General Discussion

Experiments I and II show that active immunization against the paternal strain significantly lowers the decidual cell response at implantation. Following the use of different procedures for immunization, Experiment II has suggested the interesting possibility that the response is dependent on a particular kind of immunity. Immunization with spleen cells significantly reduces decidual size, whereas immunization with skin grafts does not. Although the sera produced after skin grafting show haemagglutination titres similar to those obtained after injection of spleen cells, their effects on decidual weight appear to be different. As far as the decidual cell response is concerned, there seem to be two kinds of humoral antibody.

This view is supported by the observation that the regressions of decidual size on antibody titre, are significantly heterogeneous when we compare the different methods of immunization. Increasing titres of humoral antibodies induced by spleen cell injections progressively reduce decidual weights. Increasing titres of antibodies induced by skin grafts have no such effect.

The reduction of decidual weight by the use of passively transferred isoantisera (shown in Experiment III and IV) clearly demonstrates the importance of humoral immunity. The proportional reduction in decidual weight brought about by passive transfer is approximately equal to that brought about by active immunization. Thus there is no evidence that cellular immunity is implicated.

There are several possible explanations for the reduction of decidual size. The time of implantation of the embryo may be delayed by the presence of antibodies, the decidual stimulus produced by the

blastocyst may be reduced, or the mother's ability to respond to the inducing stimulus may be impaired. Alternatively, specific immunization may directly decrease the rate of growth of the decidual tissue. It is not possible at present to distinguish between these alternatives.

The existence of two kinds of antibody, and the effectiveness of passively transferred antiserum in depressing the decidual weight, suggest a parallel with the phenomenon of immunological enhancement (see Chapter IV). An anti-tissue isoantibody raised in mice may exhibit haemagglutinating, hemolytic, cytotoxic and enhancing activity (Amos, 1962; Gorer, 1956). In tumour enhancement, the strength of an enhancing antiserum is generally measured by its ability to protect a tumour implant from rejection. It is usually considered that an antiserum of high titre is the most effective in producing enhancement (see Kaliss, 1969). The route of administration of antigen may be decisive in determining the relative amounts of enhancing or cytotoxic antibody in an antiserum (Prehn, 1959; Medawar, 1963).

If humoral antisera induced by skin grafts contain a relatively high degree of enhancing activity, one would expect that the decidua would be relatively less affected by increasing antibody titre; an interpretation that is in accord with the results.

On the other hand, it is equally plausible to speculate that 'spleen cell' immunity results in relatively high levels of enhancing antibodies. If, as several workers have suggested (Tyler, 1961; Kirby, 1967) the process of implantation is dependent on an immunological interaction between embryonic and maternal tissue, the presence of enhancing antibody might interfere with this reaction and so result in smaller deciduas. The interference, of course, might merely be mechanical.

If the decidual cell reaction is as responsive to the presence of antibodies as these experiments suggest, and if the reaction discriminates between enhancing and cytotoxic antibodies, the results may have practical importance. The degree of decidual development could be a sensitive tool for detecting the enhancing capacities of an antiserum.

Chapter VII

Conclusions

Conclusions

1. The effects of immunization on the later stages of pregnancy

In common with the majority of attempts to prejudice the outcome of a pregnancy by maternal sensitization, the experiments reported in this thesis have confirmed the efficiency of the mechanisms protecting the foetus.

Tables 7.1 and 7.2 summarize the data on the effects of allogeneic and xenogeneic immunization on an 18th day pregnancy. The tables give the ratios of the mean values for allogeneically immunized and control groups (Table 7.1), for allogeneically and xenogeneically immunized groups and for xenogeneically and control groups (Table 7.2). If immunization had no effect, the expected ratio in each case would be unity.

There is evidence from the combined data of the five experiments that allogeneic immunization causes a small but consistent specific depression of foetal weight. Foetal weights, adjusted for litter size, are lower than control groups, significantly so in two out of the four comparisons. Hyperimmunization may be necessary to produce a reduction of foetal weight, as immunization with one skin graft and a single injection of spleen cells is ineffective (Experiment V, Table 7.1).

The foetal weights of xenogeneically immunized groups fall between the allogeneically immunized and the control values, but are not significantly different from either (Table 7.2). Although some depression may be brought about by non-specific factors, it is smaller than the effect of specific immunity.

Table 7.1

Ratios of mean values in allogeneically immunized/non-immunized groups

Experiments[1]	Foetal wt.[2]	Placental wt.[2]	Litter size	No. moles/litter	Implantation no.
I[3]	0.95	0.97	0.54**	1.79*	0.79*
II	0.95*	1.02	0.90	2.00	0.90
III[4]	0.86	1.02	0.74	-[5]	0.88
IV	0.93*	1.01	1.16	1.00	0.97
V[6]	0.98	0.96	1.25	0.40	1.16

* $p < 0.05$; ** $p < 0.01$

1. I - 1st experiment Chapter II
   II - 2nd experiment Chapter II
   III - 2nd experiment Chapter III
   IV - 3rd experiment Chapter III
   V - Chapter IV
2. Adjusted means used.
3. A_2G males.
4. Neuraminidase and hyaluronidase injected in both groups.
5. Not used for foetal death comparison as enzyme injection increases mole number in the presence of allogeneic immunity.
6. Females immunized with 1 skin graft and 1 spleen cell injection only.

Table 7.2

Ratios of mean values in A. allogeneically immunized/xenogeneically immunized groups and B. xenogeneically immunized/non-immunized groups

	Experiment[1]	Foetal wt.[2]	Placental wt.[2]	Litter size	No. moles/litter	Implantation no.
A	II	0.98	1.04	1.01	1.00	0.92
	IV	0.95	0.98	1.27	2.00	1.20*
B	II	0.96	0.98	0.89	2.00	0.98
	IV	0.97	1.03	0.91	0.50	0.80

* $p < 0.05$

1. II - 2nd expt. Chapter II
   IV - 3rd expt. Chapter III

2. Adjusted means used.

The reduction of foetal weight could be due either to a continuous retardation of foetal growth or to some event early in pregnancy that influenced later development. The work on decidual weights, reported in Chapter VI, support the latter explanation.

Although placental weights are apparently independent of the decidual response, foetal weights may be affected by the diminished decidual response characteristic of allogeneically immunized females (Chapter VI). Support for this suggestion comes from the results of genetic crosses, where the decidual response on the 7th day and the foetal weight on the 18th day are positively correlated (Hetherington, 1970). Whether the effect on foetal weight is the direct consequence of changes in the decidual response, or whether both foetal and decidual weight are dependent on a third factor, is not known.

Non-specific consequences of immunization do appear to influence other features of pregnancy. The effects of allogeneic immunization on litter size, implantation number and the incidence of foetal death (moles) are evident but inconsistent. Although clearly significant results were obtained in the C57 BL x A_2G mating, the C57 BL x CBA strain combination also showed signs of disturbance. It is not known whether these effects are caused by maternal stress or by a heightening of the generalized immune response. Evidently, further experiments involving immunization should take into account the influence of non-specific factors.

Placental weights appear to be unaffected by any method of immunization.

It is difficult to account for the difference between these results and those of James (1965), who found that pre-immunization increases both placental and foetal weights. It is particularly difficult because in Experiment I the same strain combination was used. The two sets of results may not be strictly comparable, since James (personal communication) discarded all litters containing under five embryos and those containing moles. However, the hypothesis that the size of the placenta and the foetus is strongly influenced by the specific immunological status of the mother is no longer tenable.

In many genetical studies, phenomena associated with normal pregnancy have been interpreted in terms of immunological reactions between mother and foetus, to the exclusion of other equally plausible explanations such as hybrid vigour or genetic complementation. The experimental evidence that antigenic disparity between mother and offspring favours increased foetal growth and survival (Billington, 1964) has not been confirmed. Nor has the claim been substantiated that placental weight is increased in the presence of antigenic differences. Observations on the mouse (Hetherington, 1971), the deermouse (Rogers and Dawson, 1970) and man (Jones, 1968; Seppala and Tolonen, 1970) have shown no such effect. It would seem that the usual tendency for F_1 placentae to be heavier is due to conventional hybrid vigour.

Reports of increased trophoblastic invasion in the presence of antigenic disparity (Billington, 1965) have also failed to find support (Clarke, 1969 and above).

However, in rare and abnormal situations of pregnancy maternal-foetal incompatibility may have strong immunological consequences.

The death of goat/sheep hybrid embryos may be the result of immunological damage to the placenta (Dent et al., 1971). In man Rh incompatibility and ABO blood group incompatibility lead to haemolytic disease of the foetus (see McConnell, 1969).

In recent years there have been several speculations based on the hypothesis that antigenic disparity between mother and foetus would favour embryonic survival. These have included explanations of the maintenance of histocompatibility polymorphisms (B. Clarke and Kirby, 1966), of the human sex ratio (Kirby et al., 1967), of hybrid vigour (Billington, 1964), of the difference in growth rate between the sexes (Ounsted and Ounsted, 1970), and of the effects of parity on placental and foetal weight (Jones, 1968; Warburton and Naylor, 1971). There is now little evidence to support such hypotheses.

2. The immunological susceptibility of the early foetus

The experiments reported in this thesis indicate that while the protective mechanisms preventing foetal rejection are clearly quite adequate for the established foetus in normal pregnancy, there is reason to believe that at earlier stages of development the embryo is potentially more vulnerable to immunological damage.

The results of Chapter III have shown that increasing placental permeability in allogeneically immunized females specifically increases early foetal death although it does not affect foetal weight on the 18th day of pregnancy. The attempts to prevent the formation of enhancing antibodies by splenectomy in allogeneically immunized females (Chapter IV) failed to prejudice the fate of the established foetus but significantly increased the incidence of early foetal death.

Chapter VI has shown that a significant reduction in the amount of maternal decidual tissue occurs in females specifically immunized to the paternal antigens of their embryos. The exact function of the decidual tissue is not known but an immunological protective role has been suggested. Experiments with pre-immunized mice show that blastocysts are destroyed when transplanted to extrauterine sites, yet develop normally when transferred to the uterus (Kirby et al., 1966). Since the implanting embryo is responsible for the induction of the decidual response, the effects of passively transferred antisera indicate some sort of specific reaction between maternal tissue and the tissue of antigenically foreign embryos. Some other recent experiments also support a protective role for the decidua. Allogeneic skin grafts transplanted to the uterus of female rats during the preimplantation stage of pregnancy (when decidual tissue develops beneath the grafts) survive for a significantly longer time than similar grafts placed in non-pregnant females (Beer et al., 1971). However, in these experiments, the decidual tissue was incapable of giving protection to grafts in the presence of sensitivity to the graft. Although the protective role of the decidua would not seem to be complete it may be sufficient for the requirements of early pregnancy.

The specific effects of antisera on the decidual response indicate that the hybrid embryo is expressing paternal antigens during implantation. However, the experiments reported in Chapter V have suggested that F_1 blastocysts and trophoblastic outgrowths in vitro are deficient in paternal antigens. If these statements are both correct, they suggest that, in the mouse, a significant increase of paternal antigenic expression occurs within

a few days. Further experiments on the precise effects of antiserum on the decidual response are needed before a firm conclusion can be attempted. We need to know exactly when passively administered immune serum is acting, the time course of decidual growth in actively immunized animals, and the comparative embryonic development associated with the smaller deciduas.

The importance of the immunization procedure has been clearly demonstrated by studies on the decidua. Active immunization with allogeneic spleen cells influences the decidual response dramatically whereas immunization with skin grafts has no demonstrable effect. The use of passively transferred isoantiserum indicates that humoral rather than cellular components are responsible for the response. The evidence for two kinds of humoral antibody (Chapter VI) suggests the participation of enhancing antibody. Recent work by Ralph et al. (1972) on the types of antibody produced during pregnancy have suggested a promising candidate for this enhancing factor. They have shown that the recently isolated immunoglobulin IgG3 is preferentially synthesised during pregnancy at the expense of IgM. They suggest that IgG3 could prevent complement fixing cytotoxic maternal antibody (IgM, IgG2) and sensitized maternal lymphocytes from killing foetal or placental tissue. IgG3 immunoglobulin has been shown not to fix complement and to be preferentially transported to the foetus (Grey et al., 1971; Gitlin, 1971).

3. A summary of the mechanisms protecting the foetus

In the light of the experiments described here, and of other recent work it would seem desirable to attempt a summary of foetal protection.

The existence of physical barriers between the mother and embryo, namely the trophoblast and possibly also, earlier in pregnancy, the zona pellucida and the decidual cell reaction, limits maternal-foetal antigenic exchange and minimizes the consequences of maternal cellular mechanisms.

Because these barriers are incomplete, the circulation of the mammalian mother receives small doses of foetal antigens. These antigens cause the formation of conventional cytotoxic anti-foetal antibodies, sensitized lymphocytes, and enhancing antibodies. These latter antibodies protect the foetus by attaching to trophoblast, preventing sensitized maternal lymphocytes and cytotoxic antibodies from damaging foetal cells. The trophoblastic barrier thus serves the dual function of limiting antigenic contact and acting as a target site for the attachment of enhancing antibody. The incomplete nature of the trophoblastic barrier and the weak antigenicity of trophoblast cells could well be essential for the production of this type of immunity.

Enhancing antibodies may well have evolved to meet the immunological problems of viviparity. There seems to be no obvious selective advantage in their demonstrated action on tumour growth. It would be interesting to discover if enhancing antibody is present in animals that are not viviparous.

Although the foetus appears to be isolated from the effects of maternal cellular immunity it is not isolated from some anti-foetal humoral components of the maternal immunological response.

The placenta is a barrier to the passage of most maternal immunoglobulins but IgG 7S immunoglobulins are readily transferred

across it (Cann et al., 1951; Gitlin and Morphis, 1969). It should be noted that, in animals where transmission is by the yolk sac, there is no evidence of selectivity (see Brambell, 1970). However, in general, the yolk sac route is of less importance than later colostral transmission (Brambell and Halliday, 1956).

Enhancing antibody has generally been considered to act at the trophoblastic barrier. There seems, however, no reason why it should not also pass through the placenta into the conceptus and frustrate the action of any cytotoxic antiserum which has managed to pass the first barrier.

Study of the immunological development of the embryo itself could provide some clues about how embryonic cells escape damage from IgG 7S antibodies. On the one hand, the foetus appears capable of humoral immunoglobulin synthesis (see Adinolfi and Wood, 1969; Solomon, 1971) and it possesses competent cellular immunity as shown by its capacity to reject skin grafts in utero (Silverstein and Lukes, 1962). On the other hand, there have been reports of foetal inability to produce some components of complement and of the inefficiency of foetal phagocytes.

IgG 7S immunoglobulin consist of two major components, cytotoxic antibody and cytophilic antibody, both of which need the participation of other factors to cause cell damage.

Cytotoxic antibody requires complement to lyse cells. It appears that although mammalian foetuses produce some complement, the levels are relatively much lower than in adults and the production of some components is deficient until late in foetal life. The concentration of complement in newborn humans is half that in the mother (Fireman

et al., 1969; Gewurz et al., 1968). Foetal and newborn lambs have no complement in early pregnancy and very little until after birth (Rice and Silverstein, 1964). Complement is present in foetal pigs at 40 days, but the level is much lower than that in the adult, and component C_4 is extremely low until birth (Day et al., 1969).

In mammals, the several components of complement in the presence of specific antibody act sequentially to produce cell lysis. Only one of the components need be absent, or present in inadequate amounts, for the complement system to be ineffective. Lack of an effective system could protect the embryo from maternal anti-foetal cytotoxic antibody.

If protection operates in this way, it is necessary that maternal transfer of complement to the foetus does not occur. In the few cases in which it has been studied, the placenta has been found to be impermeable to complement (Tachibana and Rosenberg, 1966).

In this context, it should be noted that although the maternal antibodies responsible for rhesus and ABO haemolytic disease are 7S IgG immunoglobulins, haemolysis of red blood cells is not dependent upon complement (Wang and Desforges, 1971).

Cytophilic antibody can only kill cells with the help of the phagocytes. The antibody acts by attaching to specific foreign cells and the complex is then engulfed by the phagocyte and destroyed. In foetal rats, Reade and Jenkin (1964) have found that although phagocytes are present and are capable of engulfing foreign particles coated with appropriate antibody, they have no bactericidal capacity, a property readily manifested by adult phagocytes. The phagocytic activity of leucocytes from premature babies has been shown to be very

inefficient *in vivo* (Gluck and Silverman, 1957).

It is suggested, therefore, that the apparent lack of complement and the inefficiency of phagocytes in the foetal system could act as a last resort or fail-safe mechanism. This idea has been formulated in a review article published by Dr. C.M. Hetherington and myself (see Appendix 7).

Knowledge of the protective mechanisms of the early foetus before the formation of the trophoblastic barrier are less well understood than those of the established foetus. The deficient antigenicity of the embryo may well be a contributing factor. IgG cytotoxic antibody only kills target tissue with a high density of surface antigenic sites (Linscott, 1970; Humphrey and Dourmashkin, 1969), and may therefore not affect the early embryo. Although the mechanisms are not known, the decidua, and earlier the *zona pellucida*, may function to some degree as anatomical barriers. The results of splenectomy in sensitized mothers suggest that the presence of enhancing antibody might also play a role in protecting the early embryo. Pregnant mice show an increase in serum IgG antibody synthesis at day 5 after immunization (Ralph *et al.*, 1972). Considerable levels are present by day 7.

In my experiments a reduced viability of F_1 blastocysts was caused by humoral antibody. The experiments of James (1969) also indicated that humoral antibodies as well as cellular components affect the development of blastocysts *in vitro*. However, Vanderputte and Sobis (1972) found that the immunological reaction inhibiting the outgrowth of allogeneic blastocysts *in vivo* was based on a cell-mediated mechanism. Transferred humoral antibodies were not active. As Vanderputte and

Sobis point out, blastocysts may be sensitive to humoral antibodies only in the presence of heterologous complement. The presence of mouse complement and antibodies may be ineffective in the natural situation of pregnancy. Humoral rather than cell-mediated mechanisms appear to be responsible for lowered decidual response.

Figure 1 is an attempt to summarize foetal protection. If it is representative of the truth we must conclude that the development of viviparity has demanded the evolution of several protective systems. Among them are the presence of the trophoblastic and other anatomical barriers, the low density or masked surface antigens of the foetus, the semi-permeability of the placenta, the induction of enhancing "barrier" antibody, and possibly, the retarded development of some subsidiary immunological capacities of the foetus.

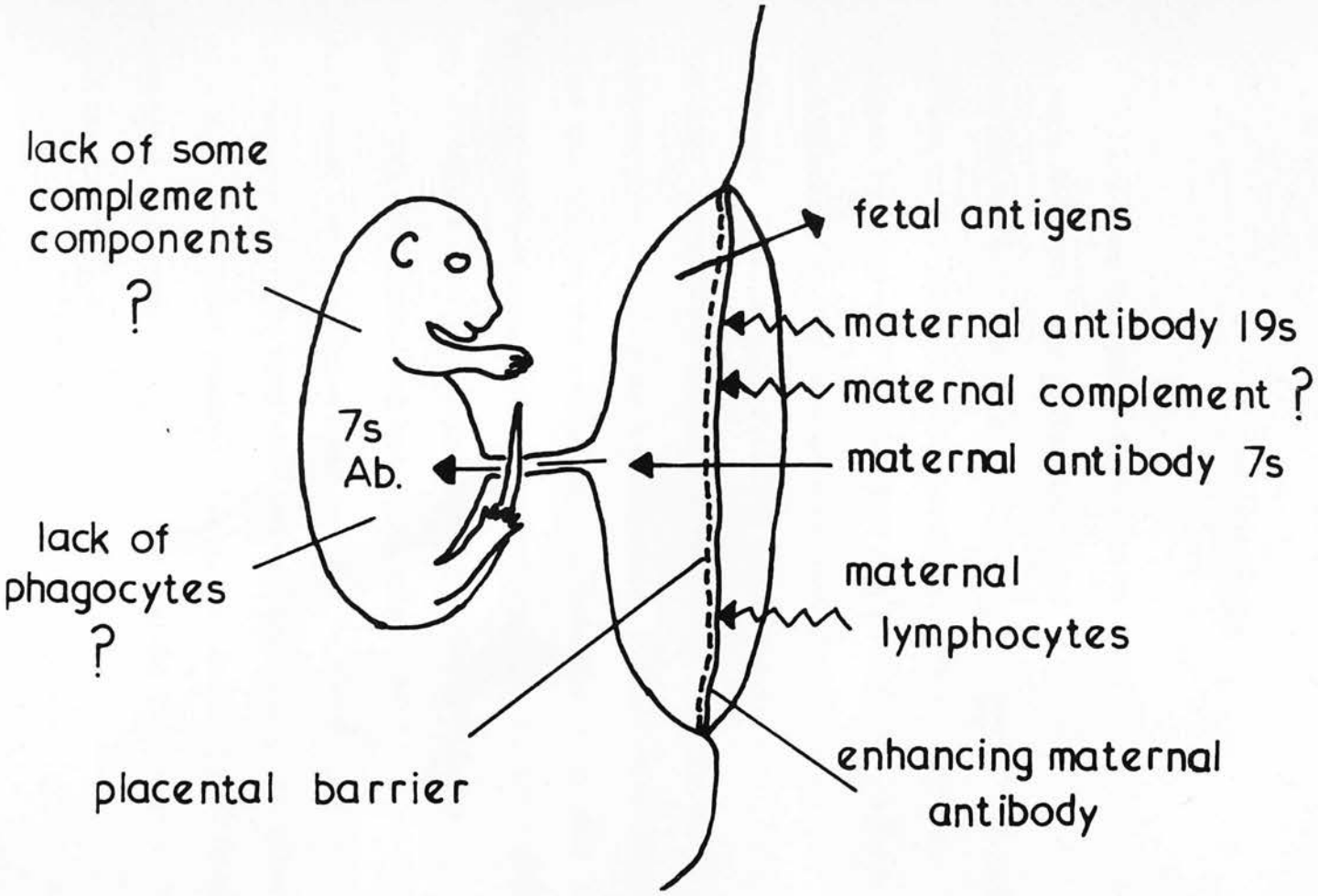


Figure 1. A SUMMARY OF FOETAL PROTECTION

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Appendix 1

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FACTORS AFFECTING THE GROWTH OF TROPHOBLAST TRANSPLANTED TO THE TESTIS

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Despite the probable existence of a protective layer around trophoblastic cells (Bradbury, Billington & Kirby, 1965; Currie & Bagshawe, 1968), there is evidence suggesting that antigenic diversity between mother and foetus has immunological consequences (Billington, 1964; McLaren, 1965; James, 1965). Billington (1965) reported that trophoblast transplanted to the testis of mice genetically dissimilar from the donor was more invasive than trophoblast transplanted to genetically similar mice. The present work was designed to investigate such an effect of genetic difference and, in particular, to see whether it was due: (1) to a response by the testis to a foreign trophoblast, (2) to a response by the trophoblast to a foreign testis, or (3) to a combination of both. In order to distinguish between these possibilities, transplants were carried out in various combinations between two strains of mice and their F_1 hybrid.

The initial series of experiments (1 to 4) was designed to investigate the first possibility and to determine if prior immunization of the recipient altered the degree of invasion. The recipient mice were all CBA males aged between 10 and 20 weeks. Half of them were grafted with two full thickness skin grafts from C57 Bl males with an 11-day interval between grafts (Gottfried & Padros, 1959). Transplantation was made to the right testis, using 7½-day-old ectoplacental cones of two kinds, either CBA or hybrid F1 (C57 Bl ♂ × CBA ♀) (for technique, see Kirby, 1962). The ectoplacental cones obtained from any one female were distributed equally amongst the grafted and ungrafted mice as far as this was possible. Recipient animals were killed 8 days after transplantation. The degree of invasion was estimated in two ways: from the difference in weight between the treated right testis and the control left testis, as in Billington (1965), and by dissecting out and weighing the trophoblastic nodules (see Table 1).

No significant differences were found between these first four experiments. This result differs from that of Billington (1965), who reported that Balb/C trophoblast grew to a larger size in C57 Bl testis than in Balb/C testis. Furthermore, in these experiments, prior immunization of recipients against donor antigens did not significantly affect trophoblastic invasion. Assuming that placental size depends on the extent of invasion by the trophoblast, this result is not in agreement with the finding of James (1965) who reported that prior immunization to maternal antigens resulted in a significant increase in placental size.

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During transplantation it was noticed that ectoplacental cones varied considerably in size, both within individual females and, in particular, between different females. In Exps. 5 to 10, cones were therefore measured before transplantation. As 7½-day ectoplacental cones are roughly triangular in shape, the length from the tip of the triangle to the base was measured as representing the overall size.

The second series of experiments (5 to 8) was designed to investigate the possibility that the trophoblast responds to foreign antigens present in the testis. Recipient mice were either hybrid (CBA ♂ × C57 Bl ♀) or C57 Bl males and were injected with either CBA or hybrid 7½-day ectoplacental cones.

TABLE 1

GROWTH OF TROPHOBLAST TRANSPLANTED TO THE TESTIS

Series	*Donor*	*Recipient*	*No. of mice (no. of invasions)*	*Mean wt of experimental − control testis ± S.E. (mg)*	*Mean wt of dissected trophoblast ± S.E. (mg)*
1	Hybrid (CBA♀ × C57 Bl♂)	CBA	14(13)	18·0 ± 8·7	17·6 ± 4·4
2	Hybrid	CBA immunized	15(12)	20·8 ± 6·9	18·4 ± 6·1
3	CBA	CBA	14(12)	23·4 ± 4·9	24·5 ± 4·6
4	CBA	CBA immunized	13(11)	14·6 ± 2·3	16·6 ± 3·1
5	CBA	Hybrid	16(14)	8·8 ± 2·5	11·3 ± 4·0
6	CBA	C57 Bl	13(11)	9·4 ± 1·9	4·7 ± 1·3
7	Hybrid	Hybrid	12(10)	14·0 ± 5·1	12·6 ± 3·6
8	Hybrid	C57 Bl	14(10)	12·2 ± 4·5	11·8 ± 3·9
9	Hybrid	CBA	14(14)	29·9 ± 6·7	29·6 ± 5·4
10	Hybrid	CBA immunized	13(13)	27·6 ± 4·8	30·5 ± 5·7

No significant differences were found between these four experiments (5 to 8) (see Table). There is, thus, no evidence in these experiments that trophoblastic growth is enhanced by the presence of foreign antigens in the testis.

Transplantation of hybrid donor tissue into non-immunized and pre-immunized CBA recipients was then repeated (Exps. 9 and 10). Results (see Table 1) supported the previous finding that trophoblastic invasion is unaltered by prior immunization of the recipient.

A significant positive correlation was found between ectoplacental cone size and the degree of trophoblast invasion (Spearman's Rank Correlation Method, $P = 0{\cdot}001$). Measurements of cones varied from 1·6 to 8·8 mm, with a mean of 4·0 mm. As the mean ectoplacental cone size in each series of transfers was similar, correction of the data to a standard 5 mm cone size had little effect on mean trophoblast weight. The exception was in Exp. 9 where the mean trophoblastic weight was slightly reduced.

Billington (personal communication) did not measure ectoplacental cone size or distribute cones obtained from one female randomly between the different series of transplantation. Although he made several inter-strain transfers, those of Balb/C into C57 Bl testis provided the only significant example of increased

invasion in the presence of genetic diversity (Billington, 1965). The results of these experiments suggest that the ectoplacental cones from the two Balb/C females used could have been larger than average.

A second factor contributing to the variability in trophoblastic growth was the different capacity of the three recipient testes (CBA, CBA × C57 B1 and C57 B1) to support growth of both CBA and hybrid donor tissue ($P < 0{\cdot}05$). CBA testes provided the most favourable environment, and C57 B1 the least favourable. A similar analysis comparing the ability of the two donor tissues (CBA and hybrid) to proliferate in all testes was not significant ($P > 0{\cdot}5$).

These results are interpreted as suggesting that factors other than immunological influences are determining the growth of 7½-day-old ectoplacental cones transplanted to the testis. The size of donor tissue transplanted and the capacity of the recipient tissue to support growth influence the degree of invasion obtained.

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Appendix 2.1

18th foetal and placental weights in allogeneically immunized, non-immunized and inbred C57 BL mice - Expt. I.

Allogeneically immunized C57 BL x A_2G

Mean Foetal wt. mg.	Mean Placental wt. mg.	Litter size no.	Moles no.	Serum antibody titre of ♀
946	173	1	0	-
988	-	2	5	$65,536^{-1}$
803	-	7	0	-
837	-	2	5	$16,384^{-1}$
1095	-	3	0	-
989	-	1	4	256^{-1}
784	138	3	0	128^{-1}
736	137	4	1	$2,048^{-1}$
791	112	6	0	256^{-1}
819	134	1	0	128^{-1}
826	138	5	2	-
977	107	3	0	128^{-1}
803	132	8	1	128^{-1}
813	142	5	0	16^{-1}
915	166	5	4	16^{-1}
1113	157	1	7	$16,384^{-1}$
936	139	7	0	$16,384^{-1}$
954	140	8	0	$1,024^{-1}$
662	139	5	0	$1,024^{-1}$
841	159	6	0	$2,048^{-1}$
1037	155	2	0	-
905	156	3	0	$2,048^{-1}$
863	185	2	5	$8,192^{-1}$
668	177	3	3	$8,192^{-1}$
803	120	9	0	16^{-1}

- Not weighed or tested

Non-immunized C57 BL x A_2G

Mean Foetal wt. mg.	Mean Placental wt. mg.	Litter size no.	Moles no.
899	138	8	0
864	104	9	0
833	139	5	1
932	139	6	2
938	169	9	2
888	138	8	1
934	144	6	1
852	155	8	0
911	152	6	0
943	125	6	1
910	-	11	0
893	-	9	0

- Not weighed

Inbred C57 BL x C57 BL

Mean Foetal wt. mg.	Mean Placental wt. mg.	Litter size no.	Moles no.
799	112	8	1
746	121	8	0
819	138	3	0
658	107	6	0
822	105	4	0
694	123	6	2
714	105	9	0
746	105	9	0
760	120	5	0
632	110	5	4
733	105	3	2
656	105	3	5
705	95	9	0

Appendix 2.2

Foetal and placental weight in allogeneically immunized, xenogeneically immunized and non-immunized C57 BL mice - Expt II

Group	Mean Foetal wt. mg.	Mean Placental wt. mg.	Litter size no.	Moles no.
Allogeneically immunized	873.6	111.47	9	0
	884.5	129.73	7	0
	873.4	128.21	9	0
	875.3	122.91	7	0
	872.6	123.52	5	2
	833.3	120.64	7	0
Xenogeneically immunized	937.8	111.70	9	0
	930.4	127.27	7	1
	973.2	118.17	7	1
	918.4	127.27	7	0
	940.4	126.83	8	2
	822.9	113.70	8	0
	675.6	95.78	9	1
	922.1	127.96	3	5
Non-immunized	943.0	113.31	8	0
	997.7	118.10	8	1
	952.2	112.47	8	0
	850.5	117.21	9	1
	886.6	129.34	7	0
	943.9	118.40	10	0
	922.3	111.11	7	1
	831.8	122.00	9	0
	912.7	124.84	6	1
	909.2	126.47	9	0

Appendix 2.3

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THE EFFECTS OF MATERNAL PRE-IMMUNIZATION ON PREGNANCY IN THE MOUSE

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Summary. Before mating with males of the A_2G or CBA inbred strains of mice, C57BL females were immunized against either the paternal strain antigens (allogeneic) or *Peromyscus* species antigens (xenogeneic). Evidence is presented which indicates that, contrary to earlier reports, pre-immunization has no effect on placental size, but may reduce foetal weight and litter size. The rôles of specific immunity and non-specific 'stress' are discussed.

INTRODUCTION

During the last 15 years, there have been conflicting reports concerning the effect on pregnancy of maternal pre-immunization against the paternal strain antigens. Several experiments have indicated that pre-immunization does not prejudice foetal survival (Mitchison, 1953; Heslop, Krohn & Sparrow, 1954; Medawar & Sparrow, 1956; Woodruff, 1957; Hasková, 1961), whereas Breyere & Sprenger (1969) and Currie (1969) have provided evidence to the contrary.

There have been suggestions that antigenic differences between the mother and her foetus have an influence on placental and foetal growth (Billington, 1964; James, 1965, 1967). Billington (1965) reported that trophoblastic invasion in an extra-uterine site tended to be greater when transplants were made between two inbred strains of mice than when they were made within strains. Clarke (1969), however, failed to detect any differences in trophoblastic invasion between isogeneic and allogeneic transfers using other strain combinations. Moreover, there was no effect of pre-immunization of the recipients against the donor-strain antigens. James (1967) found that placental and foetal weights in the mouse were increased by immunizing the female to paternal antigens and decreased by rendering her tolerant of them. The present experiments were carried out to re-examine the effect of maternal pre-immunization on placental and foetal weights and other aspects of pregnancy.

MATERIALS AND METHODS

The first experiment utilized the same strain combination as that used by James (1967). In the second experiment, a different paternal strain was used and an additional group of females was immunized xenogeneically to a *Peromyscus* species. This latter group was included to investigate the effect of a

general non-specific heightening of the immune response, and the effect of stress experienced by the females during the immunization procedure.

Experiment 1

C57BL female mice, aged approximately 6 weeks, were divided into three groups. The first group was immunized against A_2G male mice either with five spleen-cell injections (a half-spleen equivalent each), or five spleen-cell injections and two skin grafts, or two spleen-cell injections and two skin grafts. All treatments were given at fortnightly intervals. Full thickness skin grafts were applied using the method of Gottfried & Padnos (1959). The degree of immunity was measured by the human serum dextran technique (Gorer & Mikulska, 1954). The mice were then weighed, and mated to A_2G males. The second group was left untreated but also mated to A_2G males. The third group was untreated and mated to C57BL males.

A fourth group of C57BL female neonatal mice was injected with either 5 to 8 million A_2G spleen cells or with 16 million F_1 C57BL female $\times$ A_2G male spleen cells to render them tolerant to the paternal strain. Injections were made into the anterior facial vein within 24 hr of birth.

All mice were killed 17 days after the copulation plug was found. Embryos and placentae were removed from the uterus, blotted and weighed to the nearest milligram on a torsion balance. The size of a litter (live foetuses) and the number of deaths during early and middle pregnancy ('moles') were recorded. The reciprocal mating, A_2G females $\times$ C57BL males, was not studied.

Experiment 2

C57BL female mice, aged approximately 10 weeks, received three spleen-cell injections (a half-spleen equivalent each), and two skin grafts from either CBA males or from *Peromyscus polionotus polionotus* (the deer mouse). A control group was not immunized. All females were mated to CBA males and their embryos were recovered as previously described. This experiment was carried out in a different laboratory from that in which Exp. 1 was conducted and utilized a different population of mice.

RESULTS

Experiment 1

The inbred controls had significantly smaller foetuses and placentae than the two outbred groups ($P<0{\cdot}001$; see Table 1); this finding is in agreement with McCarthy (1965) and McLaren (1965). No significant difference was found, however, between the immunized and non-immunized mothers in the weight of the hybrid 17½-day-old foetuses and placentae. Nevertheless, immunization appeared to have adverse effects on the outcome of a pregnancy. It significantly decreased the size of a litter ($P<0{\cdot}01$) and among those females whose litters contained early and middle deaths ('moles'), there were larger numbers of 'moles' within the immunized mothers ($P<0{\cdot}05$, Fisher's exact test). The total number of implantations in a litter (live embryos + 'moles') was significantly lower in immunized females than in non-immunized or in inbred controls ($P<0{\cdot}05$; see Table 2). The number of young in a litter is known to

TABLE 1

COMPARISON OF MEAN $17\frac{1}{2}$-DAY FOETAL AND PLACENTAL WEIGHTS IN IMMUNIZED, NON-IMMUNIZED AND INBRED MOTHERS IN EXP. 1

Group	*No. of litters*	*Mean litter size*	*Mean foetal wt (mg ± S.E.)*	*Adjusted* mean foetal wt (mg)*	*Mean placental wt (mg ± S.E.)*	*Adjusted* mean placental wt (mg)*
Immunized C57BL × A_2G	25	4·1	876 ± 23·6	853	145·3 ± 4·6	140·6
Control non-immunized C57BL × A_2G	12	6·1	899 ± 10·3	899	140·3 ± 5·5	144·9
Inbred C57BL × C57BL	13	6·0	729 ± 16·9	730	111·6 ± 3·1	112·5

* Individual within-group regression coefficients used to adjust foetal and placental weight means to a constant litter size.

TABLE 2

COMPARISON OF NUMBER OF EARLY AND MIDDLE DEATHS OR 'MOLES' IN EXP. 1

Group	*No. of litters**	*Mean litter size*	*Females with three or more 'moles'/ litter*	*Females with < three 'moles'/ litter*	*Mean no. 'moles'/ litter*	*Embryos + 'moles'/ litter*
Immunized C57BL × A_2G	34	4·2	11	7	3·4	5·8
Control non-immunized C57BL × A_2G	25	6·5	2	9	1·9	7·3
Inbred C57BL × C57BL	14	6·1	2	4	2·5	7·0

* Litter numbers larger than in Table 1 as not all litters used for $17\frac{1}{2}$-day analysis.

TABLE 3

COMPARISON OF MEAN $17\frac{1}{2}$-DAY FOETAL AND PLACENTAL WEIGHTS IN ALLOGENEICALLY IMMUNIZED, XENOGENEICALLY IMMUNIZED AND NON-IMMUNIZED MOTHERS IN EXP. 2

Group	*No. of litters*	*Mean litter size*	*Mean foetal wt (mg ± S.E.)*	*Adjusted* mean foetal wt (mg)*	*Mean placental wt (mg ± S.E.)*	*Adjusted* mean placental wt (mg)*
Allogeneically immunized C57BL × CBA	6	7·3	869 ± 7·3	869	122·7 ± 2·6	122·5
Xenogeneically immunized C57BL × CBA	8	7·2	890 ± 34·2	884	118·6 ± 4·0	117·5
Control C57BL × CBA	10	8·1	915 ± 15·6	918	119·3 ± 2·0	119·6

TABLE 4

COMPARISON OF NUMBER OF EARLY AND MIDDLE DEATHS OR 'MOLES' IN EXP. 2

Group	*No. of litters*	*Mean litter size*	*Females with three or more 'moles'/ litter*	*Females with < three 'moles'/ litter*	*Mean no. 'moles'/ litter*	*Embryos + 'moles'/ litter*
Allogeneically immunized C57BL × CBA	6	7·3	0	1	0·3	7·7
Xenogeneically immunized C57BL × CBA	8	7·2	1	4	1·1	8·4
Control C57BL × CBA	10	8·1	0	4	0·4	8·6

affect placental and foetal weights (Healy, McLaren & Michie, 1960). Regression analysis allows these weights to be corrected to a standard litter size (5·4 in this case). Regression of foetal weight on litter size ($b = -14{\cdot}80$; $0{\cdot}1 > P > 0{\cdot}05$) is just below significance but regression of placental weight on litter size is significant ($b = -4{\cdot}20$; $P = 0{\cdot}01$). Although the correction for litter size reduces the mean foetal weight of the immunized group where litter sizes were small, the adjusted means of the control and immunized groups are not significantly different ($P > 0{\cdot}05$). This result is not in agreement with James (1967) who found an increase in placental and foetal weight after maternal immunization. The data in Table 1 show, if anything, a decrease.

The degree of immunity of immunized females as measured by the human serum dextran technique was extremely variable and could not be correlated with the mode of immunization. Haemagglutination titres ranged from 1/16 to 1/16,384. Litter sizes, foetal and placental weights and the number of 'moles' in a litter could not be correlated with the rejection times of the second immunizing skin grafts ($6{\cdot}0 \pm 2$ days), or with the titres of circulating antibody.

Of the forty-eight C57BL female mice that were injected with A_2G male spleen cells, all but two developed runt disease. Varying degrees of the typical symptoms (cessation of growth and development, the presence of diarrhoea, sparse hair from about 2 weeks of age) were followed at varying intervals by death. The two survivors received skin grafts at 8 weeks but normal rejection times (10 ± 2 days) showed the absence of tolerance. The sixty-four neonates receiving F_1 (C57BL female $\times$ A_2G male) spleen cells in an attempt to overcome runt disease also showed no tolerance. Ten individuals grafted with A_2G male skin at maturity showed rejection times that were normal.

Experiment 2

The results (Tables 3 and 4) were essentially similar to those of the first experiment. Analysis showed that the mean F_1 foetal weights from mothers specifically pre-immunized to the paternal antigens were, in this case, significantly smaller than those from control non-immunized mothers ($P < 0{\cdot}02$). Females immunized against the non-specific antigens of *Peromyscus polionotus polionotus* gave a mean foetal weight intermediate between the allogeneically immunized and non-immunized groups, but was not significantly different from either of them. Placental weights were similar in all three groups. Although the litter sizes and the total number of implantations were lower in the two immunized groups, the differences were not significant. The overall number of 'moles' was too small for a valid comparison to be made (Table 4). As in Exp. 1, the regression of foetal weight on litter size was not significant ($b = -10{\cdot}73$; $P > 0{\cdot}05$) and the regression of placental weight was significant ($b = -2{\cdot}1$; $P < 0{\cdot}05$). Correction of foetal and placental weights to a standard litter size of 7·5 reduced the significance of the difference in foetal weight between specifically immunized and non-immunized females ($P = 0{\cdot}05$).

DISCUSSION

The results of the two experiments indicate that maternal immunization to

specific paternal antigens does not enhance placental and foetal growth. Indeed, both experiments show no effect on placental growth but a reduced foetal growth after allogeneic immunization, although this was significant only in Exp. 2. These results therefore conflict with those of James (1965, 1967), who found an increase in placental and foetal weight after maternal immunization. Both of the present experiments suggest that immunization reduces litter size, although the effect was significant only in Exp. 1. This was not apparent in the experiments of James, who did not include litters containing less than five young, and litters containing 'moles'. The present findings are in agreement with those of Breyere & Sprenger (1969) who reported a decreased litter size at birth in C57BL females specifically immunized against DBA/2 sarcoma, and mated to DBA/2 males but no decrease when C57BL females were immunized non-specifically to C_3H adenocarcinoma. The present findings in Exp. 2 also indicate that non-specific xenogeneic immunization does not reduce foetal weight significantly, but some effect of stress or the raising of a general immune response on foetal weight and litter size cannot as yet be excluded.

Attempts to induce tolerance to A_2G spleen cells in C57BL female neonates failed because of the occurrence of fatal runt disease (Exp. 1). James (personal communication) found only a 5% incidence of runt disease in this strain combination using similar doses of spleen cells.

Billingham & Brent (1959) found the A/C57BL strain combination the least liable to produce tolerance of many strains tested. They obtained low tolerance in the A into C57BL combination and in the reciprocal combination (C57BL into A), they found 100% fatal runt disease. The A_2G strain was derived about fifty generations ago from the A strain and is unlikely to have acquired many changes at the histocompatibility loci during this time. This has been confirmed by Davies (personal communication) who has typed the histocompatibility loci of the A_2G strain and found them similar to the A strain.

C57BL neonates injected with F_1 (C57BL females $\times$ A_2G males) spleen cells should not be capable of responding to the antigens of the A_2G strain. Nevertheless, difficulty was experienced in bringing these mice through weaning. Billingham & Brent (1959) also report reactions to F_1 cells in the C57/A strain combination.

No satisfactory explanation can be found to account for the difference in the incidence of runt disease between the animals in this study and those of James (1967). The possibility exists that the significantly smaller foetuses and placentae which James obtained from female mice rendered 'tolerant' to the paternal antigens could have been due in part to subclinical runt disease.

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Appendix 3.1

The effect of neuraminidase and hyaluronidase on the number of live offspring and skin graft survival between mother and offspring in Q strain mice.

Enzyme	Injection		Mean Litter No.		Graft exchange	Rejection Time (days)
	No.	Total (I.U.)	1st Pregnancy	2nd Pregnancy		
Neuraminidase	8	16	10	10		
"	"	"	3		M **→ O	7
"	"	"	8	3		
"	"	"	10	5		
"	"	"	10		O **→ M	7
"	"	"	15	10		
"	"	"	10	NT	NT	
"	6	36	9		M → O	6, 5, 7
"	"	"	10	4		
"	"	"	5		O → M	Failed
"	"	"	12		O → M	6, 6
Hyaluronidase	6	2,000*	0	15		
"	"	"	10		M → O	7, 5, 5
"	"	"	0	10		
"	"	4,500	9	9		
"	"	"	15		M → O	7, 8, 9
"	"	"	9		O → M	5
Neuraminidase and Hyaluronidase	"	16 and 2,000	7		M → O	7, 7
"	"	"	7	11		
"	"	"	10	15		
"	8	36 and 4,500	9		O → M	6, 6
"	"	"	5	3		
"	"	"	12	NT		
"	"	"	11	NT		
"	"	"	13		M → O	8, 8
Saline	8	-	18		M → O	5, 8
"	"	-	15		O → M	7, 8
"	"	-	10		M → O	7
"	"	-	8		O → M	8, 9
"	"	-	8		O → M	7, 9

* Dose given by Nathan (1960) et al. scaled down to 20 gm mouse.

** M → O = Mother to Offspring, O → M = Offspring to Mother

Appendix 3.2

The effect of neuraminidase and hyaluronidase on pregnancy in allogeneically immunized and non-immunized C57 BL mice.

A. Allogeneically immunized + enzymes.

Mean Foetal wt. mg.	Mean Placental wt. mg.	Litter size	Moles		
			Total	Early	Middle
670	137.8	3	3	1	2
877	127.9	5	0		
680	121.8	10	0		
622	103.1	7	3	2	1
783	127.4	6	0		
874	122.2	7	1	0	1
701	114.4	6	1	1	0
670	131.0	1	4	4	0
478	135.3	2	6	1	5

B. Non-immunized + enzymes

879	117.4	5	1	1	0
842	125.7	6	0		
776	133.1	8	0		
999	125.4	7	1	1	0
744	118.2	11	0		
704	111.3	3	6	1	5
762	115.4	10	1	0	1
864	118.4	6	2	2	0
923	120.4	7	0		

Appendix 3.3

The effect of hyaluronidase on pregnancy in allogeneically immunized, xenogeneically immunized and non-immunized C57 BL mice.

A. Allogeneically immunized + saline.

Mean Foetal wt. mg.	Mean Placental wt. mg.	Litter Size	Moles			
			Total	Early	Middle	(Mean wt.mg.)
902	123.2	8	0			
841	118.6	10	0			
848	119.1	8	0			
888	109.8	8	0			
819	119.2	8	0			
902	111.5	6	2	2	0	
841	103.5	7	2	0	2	(57.9)
832	109.7	9	0			

B. Xenogeneically immunized + saline.

929	114.3	8	0			
811	131.0	8	0			
915	117.7	7	1	0	1	(52.5)
917	109.8	6	1	0	1	(82.6)
1007	112.2	7	1	1	0	
820	118.1	5	0			
870	129.8	3	1	1	0	
944	115.1	9	1	1	0	
887	97.4	9	0			
1066	161.2	1	0			
920	113.2	6	1	0	1	(85.0)
841	114.9	7	0			

C. Allogeneically immunized + hyaluronidase

Mean Foetal wt. mg.	Mean Placental wt. mg.	Litter Size	Moles			
			Total	Early	Middle	(Mean wt.mg.)
928	115.0	9	2	2	0	
892	116.4	9	3	2	1	(58.3)
891	114.4	6	3	0	3	(91.6)
825	121.8	5	4	0	4	(125.1)
948	119.9	6	0			
826	100.5	6	5	0	5	(122.6)
871	127.6	7	1	1	0	
785	114.7	10	0			
872	103.5	8	1	1	0	
891	123.6	2	8	4	4	(73.9)

D. Xenogeneically immunized + hyaluronidase

793	116.1	12	0			
801	112.7	10	1	1	0	
819	125.8	2	5	0	5	(73.8)
925	104.7	9	3	3	0	
850	109.4	9	1	1	0	
923	108.8	8	0			
817	122.5	7	0			
874	116.2	10	1	1	0	
864	111.7	7	1	0	1	(74.5)

E. Non-immunized + saline

881	119.0	8	1	1	0	
967	126.6	6	4	4	0	
977	109.8	6	3	3	0	
868	109.5	8	1	1	0	
915	123.8	7	0			
956	115.7	6	0			
997	99.0	8	0			
878	102.8	7	1	1	0	
961	103.3	6	2	1	1	(26.3)

Appendix 4

The effect of splenectomy on pregnancy in allogeneically immunized and non-immunized C57 BL mice.

A. Immunization and Splenectomy

Mean Foetal wt. mg.	Mean Placental wt. mg.	Litter size no.	Moles		
			Total	Early	Middle
932	102.6	8	0		
1041	117.4	2	4	4	0
792	113.0	9	1	1	0
818	109.0	4	0		
853	117.1	5	2	2	0
884	100.5	11	1	1	0
959	105.8	5	2	2	0
979	119.1	4	1	1	0
892	112.3	8	1	1	0
862	113.7	5	2	2	0
974	97.8	8	0		
1014	108.2	5	4	4	0
918	108.8	7	1	1	0
904	101.2	8	1	1	0

B. Splenectomy only

Mean Foetal wt. mg.	Mean Placental wt. mg.	Litter size no.	Moles		
			Total	Early	Middle
960	82.0	8	0		
826	105.1	4	3	3	0
996	86.0	8	0		
967	94.6	7	1	1	0
848	114.6	6	0		
963	81.8	9	0		
922	108.4	7	1	1	0
848	119.1	9	0		
992	108.1	5	0		
975	110.2	7	0		
845	102.2	9	0		

C. Immunization only

Mean Foetal wt. mg.	Mean Placental wt. mg.	Litter size no.	Moles		
			Total	Early	Middle
832	106.9	7	0		
796	84.3	8	0		
843	107.3	8	1		1 (late)
909	108.3	7	0		
873	104.4	9	0		
867	91.4	9	0		
882	112.6	7	0		
950	97.8	9	0		
858	96.1	9	0		
908	91.8	4	1	1	0
962	125.0	7	0		
899	97.6	8	0		
907	111.1	8	1	1	0
864	105.5	7	0		

D. Controls

Mean Foetal wt. mg.	Mean Placental wt. mg.	Litter size no.	Moles		
			Total	Early	Middle
886	110.2	7	0		
891	94.3	9	0		
949	91.2	9	0		
972	131.9	3	0		
864	124.6	5	0		
857	119.7	8	3	3	0
929	98.5	7	0		
916	105.5	7	1	1	
1011	123.2	2	0		
898	135.6	3	3	3	0
850	92.9	6	0		
930	113.6	8	0		
920	107.9	5	3	3	0

Appendix 5.1

Invitro culture of inbred CBA eggs in 10% C57BL anti-CBA or 10% C57BL serum, and 10% GPS - Experiment IV.

A. CONTROL C57BL SERUM.

Date	Eggs No.	Morulae No.	Blastocysts					Trophoblastic Outgrowth			Cavitation			
			No.	%	Abnormal No.	Normal No.	Normal %	No.	% of Total Eggs	% of Blast.	48 hours Total No.	48 hours Normal No.	> 48 hours Total No.	> 48 hours Normal No.
5/1	6	4	4	66.7	2	2	50.0	3	50.0	75.0	4	2	0	-
10/1	4	4	4	100.0	0	4	100.0	1	25.0	25.0	3	3	1	1
11/1	6	6	6	100.0	0	6	100.0	6	100.0	100.0	6	6	0	-
13/1*	8	8	5	62.5	1	4	75.0	4	50.0	75.0	5*	-	0*	-
14/1	4	4	3	75.0	1	2	66.7	3	75.0	100.0	1	1	2	1
16/1	5	5	5	100.0	2	3	60.0	3	60.0	60.0	5	3	0	-
17/1	5	4	4	80.0	0	4	100.0	4	80.0	100.0	4	4	0	-
20/1	9	9	6	66.7	0	6	100.0	5	55.5	83.3	6	6	0	-
5/2	9	9	4	44.4	2	2	50.0	1	11.1	25.0	4	2	0	-
9/2	5	5	4	80.0	0	4	100.0	3	60.0	75.0	4	4	0	-
13/2*	4	3	3	75.0	0	3	100.0	3	75.0	100.0	3*	-	0*	-
TOTALS	65	61(93.8%)	48	73.8	8(16.7)	40	83.3	36	55.4	75.0	37	31	3	2

B. C57BL ANTI-CBA SERUM.

Date	Antiserum Group No.	Titre (Rank)	Eggs No.	Morulae No.	Blastocysts							Trophoblastic Outgrowth			Cavitation			
					No.	%	Angular Transformed %	Abnormal No.	Normal No.	Normal %	Angular Transformed %	No.	% of Total Eggs	% of Blast.	48 hours Total	48 hours Normal	> 48 hours Total	> 48 hours Normal
5/1	15	256^{-1} (4)	7	7	3	42.9	40.9	3	0	0	0	1	14.3	33.3	0	-	3	0
10/1	7	32^{-1} (1)	5	5	4	80.0	63.4	1	3	75.0	60.3	4	75.0	100.0	4	3	0	-
11/1	7	32^{-1} (1)	6	6	6	100.0	90.0	0	6	100.0	90.0	6	100.0	100.0	5	5	1	1
13/1*	8	512^{-1} (5)	7	7	0	0	0	0	0	0	0	0	0	-	0*	-	0*	-
14/1	8	512^{-1} (5)	4	4	1	25.0	30.0	0	1	100.0	90.0	0	0	0	1	1	0	-
16/1	8	512^{-1} (5)	3	3	3	100.0	90.0	3	0	0	0	0	0	0	2	0	1	0
17/1	8	512^{-1} (5)	7	7	4	57.1	49.1	1	3	75.0	60.0	3	42.9	75.0	2	2	2	1
20/1	58	64^{-1} (2)	10	10	5	50.0	45.0	5	0	0	0	1	10.0	20.0	5	0	0	-
5/2	15	256^{-1} (4)	13	11	5	38.5	38.4	5	0	0	0	0	0	0	1	0	4	0
9/2	1	512^{-1} (5)	5	5	2	40.0	39.2	1	1	50.0	45.0	0	0	0	2	1	0	-
13/2*	1	512^{-1} (5)	3	2	0	0	0	0	0	0	0	0	0	-	0*	-	0*	-
TOTALS			70	67(95.7%)	33	47.1		19(57.6)	14	42.4		15	21.4	45.5	22	12	11	2

* Data not included in cavitation analysis as no eggs cavitated in B.

Appendix 5.2

In vitro culture of C57 BL x CBA, C57 BL and CBA eggs in 10% C57 BL anti-CBA serum and 1% Guinea Pig Serum - Series I.

Date	Eggs	Anti-serum group	Eggs No.	Morula No.	Blastocysts					Trophoblastic Outgrowth		
					No.	%	Abnormal No.	Normal No.	Normal %	No.	% of Total Eggs	% of Blast. No.
8/12	C57 BL x CBA	18	28	25	25	89.3	1	24	96.0	-	-	-
9/12		18	17	13	13	76.5	5	8	61.5	-	-	-
15/12		25	5	5	5	100.0	-	-	-	1	20.0	20.0
16/12		25	17	14	14	82.3	0	14	100.0	10	58.8	71.4
17/12		0	8	7	7	87.5	0	7	87.5	6	75.0	85.7
Totals			75	64 (85.3%)	64	85.3	6/59	53/59	89.8	17	17/30 56.7	(17/26) 65.4
9/12	C57 BL	18	9	8	6	66.7	2	4	66.7	-	-	-
12/12		9	3	3	3	100.0	0	3	100.0	1	33.3	33.3
14/12		25	14	14	12	85.7	-	-	-	-	-	-
15/12		25	12	10	4	33.3	-	-	-	-	-	-
16/12		25	13	12	10	76.9	0	10	100.0	9	69.2	90.0
17/12		0	14	14	14	100.0	2	12	85.7	4	28.6	28.6
Totals			65	61 (93.8%)	49	75.4	4/33	29/33	87.9	14	14/30 46.7	(14/27) 51.9
9/12	CBA	18	6	4	4	66.7	4	0	0	-	-	-
12/12		9	3	3	0	0	0	0	-	0	0	-
14/12		25	24	24	22	91.7	-	-	-	-	-	-
15/12		25	8	8	8	100.0	-	-	-	8	100.0	100.0
16/12		25	22	18	10	45.5	8	2	20.0	10	45.5	100.0
17/12		0	17	7	7	41.2	5	2	28.6	1	5.8	14.3
Totals			80	64 (80.0%)	51	63.8	17/21	4/21	19.0	19	19/50 38.0	(19/25) 76.0

- Not tested

Appendix 5.2a

Invitro culture of C57BL x CBA, C57BL and CBA eggs in 10% C57BL anti-CBA serum and 10% Guinea pig serum - Series II.

Date	Group Culture	Anti-serum Group No.	Antibody Titre (Rank)	Eggs No.	Morulae No.	Blastocysts					Trophoblastic Outgrowth		
						No.	%	Abnormal No.	Normal No.	Normal %	No.	% of Total Eggs	% of Blast. No.
18/12	C57BL x CBA	4	128^{-1}(3)	13	10	10	76.9	2	8	80.0	7	53.8	70.0
19/12		4	128^{-1}(3)	5	4	3	60.0	1	2	66.7	2	40.0	66.7
20/12		4	128^{-1}(3)	3	3	3	100.0	1	2	66.7	3	100.0	100.0
22/12		56	64^{-1}(2)	6	5	5	83.3	1	4	80.0	5	83.3	100.0
23/12		3	512^{-1}(5)	9	9	5	55.6	1	4	80.0	5	55.6	100.0
Totals				36	31 (86.1%)	26	72.2	6	20	76.9	22	61.1	84.6
19/12	C57BL	4	128^{-1}(3)	8	8	7	87.5	0	7	100.0	7	87.5	100.0
21/12		56	64^{-1}(2)	10	10	10	100.0	0	10	100.0	8	80.0	80.0
22/12		56	64^{-1}(2)	6	6	4	66.7	0	4	100.0	0	0	0
23/12		3	512^{-1}(5)	10	10	10	100.0	0	10	100.0	10	100.0	100.0
Totals				34	34 (100.0%)	31	91.2	0	31	100.0	25	73.5	80.6
18/12	CBA	4	128^{-1}(3)	13	13	3	23.1	2	1	33.3	3	23.1	100.0
19/12		4	128^{-1}(3)	3	3	1	33.3	1	0	0	1	100.0	100.0
20/12		4	128^{-1}(3)	21	19	15	71.4	7	8	53.3	11	52.4	73.3
21/12		56	64^{-1}(2)	8	8	5	62.5	2	3	60.0	5	62.5	100.0
22/12		56	64^{-1}(2)	4	4	3	75.0	1	2	66.7	0	0	0
Totals				49	47 (95.9%)	27	55.1	13	14	51.9	20	40.8	74.1

Appendix 6.1

Decidual weight in allogeneically immunized, xenogeneically immunized and non-immunized C57 BL mice - Experiment I

A. Allogeneically immunized

Decidual wt. mg.	Implantation no.	Lumbar node wt. mg. (no. of nodes)	Spleen wt. mg.	Serum antibody titre (no. +ve dilutions)
2.19	9	7.5 (2)	137.8	32^{-1} (5)
2.55	12	6.0 (2)	147.2	32^{-1} (5)
1.50	5	7.1 (2)	104.6	$4,096^{-1}$ (12)
2.66	7	6.2 (2)	119.0	32^{-1} (5)
2.42	6	5.2 (2)	124.7	$4,096^{-1}$ (12)
2.05	10	5.4 (2)	108.0	16^{-1} (4)
2.10	8	5.1 (2)	130.6	$1,024^{-1}$ (10)
2.48	8	4.5 (2)	114.1	64^{-1} (6)
3.63	8	8.4 (2)	111.4	128^{-1} (7)
1.79	9	6.9 (2)	119.0	$8,192^{-1}$ (13)
1.84	8	4.3 (2)	97.8	512^{-1} (9)
2.17	6	7.2 (2)	150.2	$8,192^{-1}$ (13)
2.65	2	9.6 (2)	128.8	$1,024^{-1}$ (10)
2.10	7	5.0 (2)	116.3	64^{-1} (6)

B. Xenogeneically immunized

Decidual wt. mg	Implantation no.	Lumbar node wt. mg (no. of nodes)	Spleen wt. mg	Serum antibody titre (no. +ve dilutions)
3.98	11	11.7 (2)	145.0	$2,048^{-1}$ (11)
3.12	6	4.7 (2)	110.1	N.T.
3.51	8	3.5 (1)	117.1	$32,768^{-1}$ (15)
3.01	10	12.0 (2)	108.0	$16,384^{-1}$ (14)
3.51	9	7.0 (2)	116.0	$4,096^{-1}$ (12)
2.15	6	5.4 (1)	167.5	$16,384^{-1}$ (14)
3.11	8	8.6 (2)	122.6	$16,384^{-1}$ (14)
4.04	8	7.6 (2)	109.4	$65,536^{-1}$ (16)
4.55	10	7.7 (2)	126.0	$65,536^{-1}$ (16)
2.57	7	7.6 (2)	135.1	N.T.
3.29	9	6.2 (2)	134.6	N.T.
3.14	7	4.6 (2)	141.5	$8,192^{-1}$ (13)
2.08	6	6.6 (2)	109.7	N.T.
1.54	5	6.8 (2)	99.0	$65,536^{-1}$ (16)
3.33	9	8.6 (2)	129.8	$16,384^{-1}$ (14)
3.88	9	6.5 (2)	132.7	$32,768^{-1}$ (15)
4.38	11	9.2 (1)	141.1	N.T.

N.T. - Not tested

C. Non-immunized

Decidual wt. mg.	Implantation no.	Lumbar node wt. mg. (no. of nodes)	Spleen wt. mg.
4.09	9	4.6 (2)	128.6
3.13	10	2.6 (1)	97.1
2.80	10	2.7 (1)	121.4
4.03	10	2.1 (1)	106.7
2.67	6	5.8 (2)	123.1
3.55	6	6.2 (2)	117.3
2.60	10	5.6 (2)	109.3
2.50	8	3.8 (2)	100.0
4.13	9	2.7 (1)	99.1
3.16	11	2.6 (1)	139.0
3.56	9	4.3 (2)	111.3
3.94	8	3.0 (2)	98.7
3.06	8	5.1 (2)	103.0
2.23	8	5.0 (2)	110.9
4.21	9	4.0 (2)	123.0
3.00	8	3.5 (2)	98.8
3.00	8	N.T.	116.8

Appendix 6.2

Decidual weight in allogeneically immunized, xenogeneically immunized and non-immunized C57 BL mice - Experiment II

A. Allogeneically immunized with skin grafts and spleen cell injections

Decidual wt. mg.	Implantation no.	Serum antibody titre (no. +ve dilutions)
5.25	8	64^{-1} (6)
4.95	8	512^{-1} (9)
5.38	9	32^{-1} (5)
4.04	10	64^{-1} (6)
4.84	9	N.T.
4.52	9	256^{-1} (8)
4.60	10	128^{-1} (7)
4.34	12	512^{-1} (9)
4.44	7	N.T.
3.34	7	512^{-1} (9)
4.50	12	32^{-1} (5)
3.87	9	$2{,}048^{-1}$ (11)
3.78	9	512^{-1} (9)

B. Allogeneically immunized with spleen cell injections only

Decidual wt. mg.	Implantation no.	Serum antibody titre (no. +ve dilutions)
5.28	9	-ve (0)
4.20	7	8^{-1} (3)
4.56	10	64^{-1} (6)
4.34	10	64^{-1} (6)
5.05	10	-ve (0)
4.53	9	16^{-1} (4)
5.31	8	4^{-1} (2)
4.87	10	32^{-1} (5)
5.07	9	32^{-1} (5)
3.78	7	N.T.
4.70	8	4^{-1} (2)
4.20	6	8^{-1} (3)
4.05	8	128^{-1} (7)
3.79	7	16^{-1} (4)
5.83	8	8^{-1} (3)

C. Allogeneically immunized with skin grafts only

Decidual wt. mg.	Implantation no.	Serum Antibody Titre (no.+ve dilutions)
5.70	8	$1,024^{-1}$ (10)
6.05	11	$2,048^{-1}$ (11)
5.52	11	32^{-1} (5)
4.56	10	512^{-1} (9)
4.81	9	64^{-1} (6)
4.63	10	4^{-1} (2)
4.85	6	8^{-1} (3)
4.22	9	16^{-1} (4)
3.93	8	-ve (0)
4.78	11	N.T.
4.79	8	16^{-1} (4)
5.16	9	8^{-1} (3)
5.01	8	8^{-1} (3)
4.58	9	N.T.

D. Xenogeneically immunized

Decidual wt. mg.	Implantation no.
5.15	10
5.30	10
4.94	7
5.14	8
4.56	9
5.89	9
4.36	11
4.28	9
5.28	8
4.66	8
5.21	9
3.96	8
5.51	10
5.13	10

E. Non-immunized controls

Decidual wt. mg.	Implantation no.
4.25	6
5.81	9
5.91	9
5.00	9
5.88	8
5.57	7
4.56	8
4.34	7
5.91	8
5.20	6
5.66	9

Appendix 6.3

Decidual weights in passively immunized C57 BL mice

Experiment III

A. Immune serum (1 s.g. and 1 s.c.)		B. Control serum	
Decidual wt. mg.	Implantation no.	Decidual wt. mg.	Implantation no.
5.46	7	5.38	8
6.01	9	5.05	2
5.23	8	5.74	8
5.53	4	6.18	8
		4.80	6
		4.86	5
C. Immune serum (2 s.g. and 3 s.c.)		D. Control serum	
Decidual wt. mg.	Implantation no.	Decidual wt. mg.	Implantation no.
5.13	8	5.55	9
4.10	10	5.57	7
4.98	8	5.50	8
4.93	8	6.02	9

Experiment IV

A. Immune serum (2 s.g. and 3 s.c.)		B. Control serum	
Decidual wt. mg.	Implantation no.	Decidual wt. mg.	Implantation no.
2.78	9	4.44	9
3.67	9	3.50	4
2.33	6	3.14	7
3.75	12	3.00	9
2.88	8	3.82	11
2.86	7	3.60	5
2.88	8	3.44	9
3.50	8	3.86	7
		4.00	8
		3.29	7

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Effects of Maternal Preimmunization on the Decidual Cell Reaction in Mice

ALTHOUGH several hypotheses have been put forward to explain the success of the mammalian conceptus as a homograft[1], there is still no agreement on the exact mechanisms involved. It has been postulated that during the early stages of pregnancy the uterine decidual tissue prevents the immunological consequences of antigenic differences between mother and conceptus[2]. Recent evidence has suggested that the extent of the decidual cell reaction is reduced when there are genetic differences between mother and offspring[3]. We have therefore investigated whether the decidual cell reaction is affected by preimmunization of the mother to paternal antigens.

CBA/Fa and C57BL/Fa inbred mice which differ at the H_2 locus were used. C57BL female mice aged between 8 and 12 weeks were distributed at random into three groups. The first group was immunized allogeneically to CBA male mice, the second group was immunized to cells from *Peromyscus maniculatus bairdii* and *Peromyscus polionotus polionotus* (deer mice), to test the effects of xenogeneic immunization, and the third group was left untreated, as controls. The immunization procedure in both instances consisted of two full thickness skin grafts[4], followed by three spleen cell injections (0.5 spleen equivalent each). All treatments were given at 14 day intervals. Skin grafts of both *Peromyscus* and CBA healed satisfactorily, and second set rejection times were 6 ± 2 days for both the allogeneically and xenogeneically immunized groups. Six days after the last injection all mice were mated to CBA males. On the seventh day of pregnancy (the day of vaginal plug representing the first day) mice were killed, and serum was collected from each female for titration of antibody. Decidual tissue was dissected from the uterus, in physiological saline; no attempt was made to remove the tissue of the conceptus. The decidua from each uterus were blotted lightly, pooled and

Table 1 Decidual Weights, Lumbar Node Weights and Spleen Weights in Allogeneically Immunized, Xenogeneically Immunized and Non-immunized Female Mice

	No. of females	Mean implant number ±s.e.	Mean decidual weight (mg) ±s.e.	Lumbar nodes weight (mg) ±s.e.	Spleen weight (mg) ±s.e.
Allogeneic immunization	14	7.5±0.6	2.29±0.13	6.31±0.4	122.1±4.1
Xenogeneic immunization	17	8.2±0.4	3.25±0.20	7.31±0.6	126.2±4.2
Controls	17	8.6±0.3	3.27±0.15	3.97±0.4	119.9±2.9

weighed to the nearest 0.1 mg. The mean decidual weight per implantation site was calculated for each female.

Data were subjected to an analysis of variance. The mean decidual weight from females immunized to CBA antigens was significantly less than the mean weight of either the untreated control group or the group immunized to *Peromyscus* ($P < 0.001$, Table 1). There was no significant difference between the untreated controls and the xenogeneically immunized females. The effect of immunization with cells of the paternal strain in decreasing decidual weight is therefore not a result of a general heightening of a non-specific immune reaction nor of stress experienced during immunization.

Maternal antibody to red cells was measured by the serum–dextran method of Gorer and Mikulska[5]. Sera from allogeneically immunized females produced haemagglutination titres ranging from 1/8 to 1/4,096. Sera from xenogeneically immunized mice gave high haemagglutination titres (1/1,024 to 1/32,768) when tested against *Peromyscus* red blood cells, but showed no cross reactivity when tested against CBA antigens. The control females showed no activity with either antigen. Lumbar lymph nodes and spleens were dissected from each female at autopsy and weighed. Immunized mice (both xenogeneic and allogeneic) had significantly larger lymph nodes ($P < 0.001$) and spleens ($P < 0.05$) than controls. The mean implantation number in the allogeneically immunized mice was lower than in the other groups but the reduction was not significant (Table 1).

There are several possible explanations for the effect of immunization to paternal antigens. The time of implantation of the embryo may be delayed, the decidual stimulus produced by the blastocyst may be reduced or the mother's ability to respond to the inducing stimulus may be impaired. Alternatively, specific immunization may directly decrease the rate of growth of the decidual tissue. It is not possible at present to distinguish between these alternatives.

In previous experiments involving maternal immunization to paternal antigens Mitchison[6] found no adverse effects on the outcome of a pregnancy. James[7], however, claimed that maternal sensitization to paternal antigens increased placental and foetal weight. Clarke[8] was unable to confirm these results and found that when C57BL/Fa females were immunized to CBA/Fa antigens, placental weight was unaffected although foetal weight was reduced. This reduction, in conjunction with the reduction in decidual weight observed in this experiment, is in agreement with the observations of Hetherington[3], who found a positive association between decidual weight on the seventh day of pregnancy and foetal weight on the eighteenth day. It is not known whether the reduction in

foetal weight is the direct consequence of the reduced decidual response or whether both foetal and decidual weights are dependent on a third factor.

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IMMUNOGENETIC ASPECTS OF MATERNAL/FETAL RELATIONS

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Summary. The evidence for immunological interactions between the mammalian mother and her conceptus are reviewed, together with the current hypotheses that have been put forward to account for embryonic indifference to the maternal homograft reaction. We propose that additional factors may be important. The relative deficiency of phagocytes, and of some components of complement within the fetus, may protect it from the maternal antifetal antibody that is known to gain access to fetal tissues.

The mammalian placenta not only allows for the exchange of nutrients and excretory products vital to the maintenance of the developing fetus, but also prevents the passage of molecules and cells which are harmful to it. The existence of a close physical connection demands a mechanism to prevent the immunological rejection of the conceptus by the mother. The widespread existence of genetic polymorphism for transplantation antigens ensures that in the majority of instances antigenic differences exist between the two organisms.

It is the aim of this paper to review the evidence for immunological interactions between mother and conceptus, and to examine the mechanisms by which the fetus is thought to be protected from their adverse effects.

Several hypotheses have been suggested to account for the survival of the fetus despite its similarity to a homograft. Two, in particular, will be considered: first, the supposed presence of barriers separating the mother and her conceptus; second, the postulated occurrence of immunological enhancement. A third mechanism will be proposed: that the deficiency of some components of fetal complement and the relative inefficiency of fetal phagocytes minimize the adverse effects of maternal antibody.

In an ideal environment, the genetic constitution of a fetus is the ultimate factor limiting its growth. It is doubtful, however, whether it ever achieves its full genetic potential, for the environment in which it develops will invariably be sub-optimal. Maternal genotype and weight, parity and litter size are all known to affect fetal development (Brumby, 1960; McCarthy, 1965; McLaren, 1965; Calkins, 1967; McKeown & MacMahon, 1956). Fetal weight and placental weight, which are positively correlated, are both negatively correlated with gestation length in guinea-pigs (Ibsen, 1928) and in mice (McLaren, 1965).

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When considering the development of the conceptus it is essential to appreciate the complexity of the interactions that occur, and to consider all these variables in the interpretation of an experiment aimed at the study of any single parameter.

In many instances phenomena associated with normal pregnancy have been interpreted in terms of immunological reactions between mother and fetus, to the exclusion of other equally plausible explanations such as hybrid vigour or phenomena similar to genotypic complementation, in which each genotype compensates for the genetic deficiencies of the other.

THE EFFECTS OF GENETIC DISSIMILARITY BETWEEN MOTHER AND CONCEPTUS

Genetic dissimilarity between mother and conceptus can apparently affect the phenotypic ratios found among the offspring. A deficiency of offspring of maternal genotype has been reported in mice, for coat colour and transplantation antigens (Hull, 1964, 1969) and in rats, for transplantation antigens (Palm, 1969). In the deermouse, Carham, Birdsall & Cameron (1970) found an excess of offspring with transferrin phenotype like the mother. The mechanisms underlying these phenomena are not understood, although in some circumstances they may involve differential fertilization. In mice, spermatozoa carrying the 't' allele of the tailless (T) locus have an apparent advantage over other spermatozoa (Braden, 1958). In an inbred line of rats Michie & Anderson (1966) have suggested that continuing heterozygosity at a locus determining transplantation antigens might be due to differential fertilization.

The development of the conceptus from implantation to parturition depends both on its own genotype and on the maternal environment. F_1 placentae and fetuses in an inbred mother are usually heavier than those of inbred conceptuses (McLaren, 1965; McCarthy, 1965) and inbreeding random-bred mice reduces both fetal and placental weight on the 18th day of pregnancy (McCarthy, 1968). There are, however, a few exceptions. The placentae of JU × CBA F_1 conceptuses are lighter than those of inbred JU × JU conceptuses (Hetherington, 1971). In crosses between two species of *Peromyscus*, while the F_1 placenta is heavier than that of either parent when the female is of the one species, placental weight in the reciprocal cross is significantly lighter (Rogers & Dawson, 1970). The usual tendency for F_1 placentae to be heavier than inbred placentae led Billington (1964) to postulate that immunological factors are involved. He argued that hybrid vigour could not solely account for the larger F_1 placentae, because A_2G eggs grown in a C57BL mother gave rise to larger placentae than C57BL eggs grown in a C57BL mother, a situation where hybrid vigour is eliminated. The results were not significantly different from those obtained from hybrid matings. However, egg transfer experiments do not exclude the possibility of complementation phenomena; the mother of one inbred line perhaps being able to supply the deficiencies of a fetus from the other. In addition, the size to which a placenta develops is known to be affected by the genotype of the conceptus (McCarthy, 1965; McLaren, 1965; Hetherington, 1970) and it is not possible, from the data provided, to exclude the possibility that the observed differences are simply due to genotype.

A study involving F_1 mothers of three genotypes of mice, in which the effects of hybrid vigour of the conceptus are minimized, did not substantiate the hypothesis that placental weight was increased in the presence of antigenic difference (Hetherington, 1971). Indeed, the data of Jones (1968) on ABO(H) incompatibility in man suggested that placental weight was increased in compatible matings, while Seppala & Tolonen (1970) found no effect of the ABO(H) locus on placental weight, birth weight or gestation length. On the other hand, Towanen & Hirovanen (1970) found that the placentae of male fetuses from primiparous females were significantly heavier in incompatible matings than in compatible matings, but no similar effect was found in other parities, or among female fetuses. As the human populations that were studied are outbred, the general failure to observe a correlation between antigenic differences and placental weight lends support to the alternative hypothesis that F_1 placentae are heavier because of hybrid vigour.

Further apparent support for the hypothesis that immunological interactions might be responsible for the larger size of hybrid placentae came from an investigation into the degree of trophoblastic invasion in extra-uterine sites found within and between strains of mice differing at the H-2 locus. Billington (1965) suggested from an experiment on mice, in which trophoblast was transplanted to the testes of allogeneic hosts, that invasiveness was increased when graft and host differed antigenically. It was suggested that if placental weight depended on the degree of trophoblastic invasion, this increased invasiveness could account for the increased placental growth found in the presence of antigenic differences between mother and conceptus. Clarke (1971) and James (unpublished data), however, were unable to confirm that antigenic differences between graft and host influenced trophoblastic invasion in the mouse testis. Similarly, Koren, Abrams & Behrman (1968) found no correlation between trophoblastic invasion and antigenic differences, using the mouse kidney as the graft site.

The hypothesis that antigenic differences between mother and conceptus result in increased placental and fetal weight and the observations of James (1967; see below) on the effects of maternal immunization led Clarke & Kirby (1966) to postulate that improved fetal growth could provide a selective mechanism capable of maintaining the balanced polymorphism associated with histocompatibility loci. However, there is now little confirmed experimental data to support such a hypothesis.

While maternal/fetal incompatibility under normal conditions apparently does not affect placental weight, it does sometimes have other effects. Recent evidence from electron micrographs has suggested that the death of goat/sheep hybrid embryos may be the result of immunological damage to the placenta (Dent, McGovern & Hancock, 1971). The immunological consequences of Rh incompatibility between the human mother and fetus in the occurrence of Rh-haemolytic disease of the fetus have been well documented (see review by McConnell, 1969).

Parity increases placental weight in a number of species (Ibsen, 1928; McKeown & Record, 1953; McCarthy, 1965; Jones, 1968; Warburton & Naylor, 1971) and can apparently affect the genotypic ratios of the offspring

(Hull, 1969). Parity also affects the immunological status of the mother; *post-partum* female mice show a specific reduction in responsiveness to the transplantation antigens of the mating male (Breyere & Barrett, 1960; Breyere & Burhoe, 1963; Kaliss & Dagg, 1964).

While exhibiting this reduction in responsiveness, parous female mice nevertheless possess humoral and cell-bound immunity to the male strain antigens (Goodlin & Herzenberg, 1964; Kaliss & Dagg, 1964; Mishell, Herzenberg & Herzenberg, 1963; Soren, 1967). It has been suggested, therefore, that the effects of parity on placental and fetal weight may have an immunological basis (Jones, 1968; Warburton & Naylor, 1971). Warburton and Naylor postulated that, if placental and fetal weight increased with parity due to sensitization to paternal antigens, the parity effect should be reduced when there is a change of partners between first and second pregnancies and the second husband differs antigenically from the first. The data they produced on ten paired comparisons provided some evidence for the hypothesis that the effects of parity might have an immunological basis, and none for refuting it. However, in a study involving three different strains of mice, and comparing placental weight in uniparous females after matings with these three strains, the sire of the first pregnancy did not affect placental weight in the second pregnancy (Hetherington, 1970).

THE EFFECTS OF MATERNAL PRE-IMMUNIZATION TO PATERNAL ANTIGENS

In the normal state of pregnancy the fetus is well protected from the homograft reaction. Studies of pregnancy after maternal immunization to paternal antigens have produced conflicting results. While some workers have found that pregnancy is unaffected by the mother's immunological state (Mitchison, 1953; Heslop, Krohn & Sparrow, 1954; Medawar & Sparrow, 1956; Woodruff, 1957; Lanman, Dinerstein & Fikrig, 1962), others have reported changes in various aspects of pregnancy. James (1967) observed that pre-immunization of mice to paternal antigens resulted in increased fetal and placental weight and decreased gestation length. Clarke (1971), however, using the same combination of strains, was unable to find an effect of pre-immunization on mouse placental weight, but obtained evidence of a reduction in fetal weight and in the number of live births. Breyere & Sprenger (1969) have also reported a reduction in the number of live births. Boshier & Moriarty (1970), working with sheep, were unable to find any effect of presensitization of the ewe to the mating ram on fertility, fecundity, placental weight or fetal weight.

There is, therefore, no good evidence that either embryonic or placental growth is increased by maternal sensitization to paternal antigens in normal pregnancy. Studies of trophoblast invasiveness in the mouse testes do not indicate that this is affected by host pre-immunization to donor antigens (Clarke, 1969).

The effects of maternal tolerance to paternal antigens have also been studied. James (1967) reported that placental and fetal weights were decreased and gestation length increased in mice rendered tolerant to paternal antigens, but the effects of non-specific factors was not excluded (Clarke, 1971). Husain & Ketchel (1965) found no influence on the time of parturition in rats which had

acquired an immunological tolerance to their mates. Changes in the immunological status of the mother, either by experimental means or by changes in parity, have thus lent little support to the hypothesis that the course of pregnancy may be significantly affected by maternal immunity to paternal antigens, or retarded by maternal tolerance.

There is some evidence indicating that the expression in the offspring of paternal alleles at the immunological loci can be altered by pre-immunization. Isoantigenic differences of the immunoglobulins (allotypes) have been demonstrated in man (Grubb, 1956; Ropartz, Lenoir & Rivat, 1961; Steinberg, 1962), rabbits (Oudin, 1960; Todd, 1963) and mice (Herzenberg, Warner & Herzenberg, 1965). Heterozygous fetal rabbits in a mother experimentally pre-immunized to the paternal immunoglobulin do not produce this allotype despite the presence of the appropriate gene (Dray, 1962; Mage & Dray, 1965; Mage, 1967; Dubiski, 1967). This suppression may be prolonged, possibly life-long, while the level of the maternally inherited immunoglobulin shows a compensatory increase. Reports of a similar suppression in humans have come from Fudenberg & Fudenberg (1964) and Martensson & Fudenberg (1965). Whether a suppression of paternally acquired genes occurs in systems other than the immunoglobulins is unknown. A suppression such as this could provide some measure of protection to the fetus from the consequences of particular genetic incompatibility with the mother.

THE FETUS AS A HOMOGRAFT

Antigenic nature of the fetus

The lack of expression of the fetal antigens before birth is a possible mechanism by which the conceptus could avoid immunological rejection.

However, the antigenic nature of the fetus in the normal situation of pregnancy has been clearly demonstrated. On the other hand, the time of development, the first appearance and the nature of the antigenic expression in the early embryo has not yet been satisfactorily established.

The development of antigenicity in the early embryo has been investigated using techniques of tissue transplantation, *in-vitro* egg culture and serological methods. Simmons & Russell (1965) and Kirby, Billington & James (1966) found that mouse blastocysts transplanted to the kidney capsules of specifically pre-immunized hosts did not develop. This result suggested that blastocysts displayed antigens at their surface. *In-vitro* culture experiments have shown the presence of antigens on naked eight-cell mouse embryos (Heyner, Brinster & Palm, 1969) and on blastocysts (James, 1969). Olds (1968), using immunofluorescent techniques, claims to have detected H-2 antigens on zona-free two-cell mouse embryos but Sell, Coombs and Edwards (see Gardner & Edwards, 1968) were unable to find them on the unimplanted blastocysts.

The antigenic nature of the later embryo has been well established. Thirteen-day-old mouse embryo liver cells induce antibody production when injected into host mice (Moller, 1963a) and 9-day-old mouse embryonic tissue, transplanted into pre-immunized allogeneic hosts, undergoes classical homograft rejection (Edidin, 1964).

Whether these reactions involve transplantation antigens rather than specifically fetal or tissue specific antigens is unknown. The cytotoxic effects on the fetus of antisera resulting from hyper-immunization with malignant tissue Moller, 1961; Schlesinger, 1965) do not prove the significance of transplantation antigens, because embryonic and neoplastic tissues may have other antigenic determinants in common (Abelev, 1963; Gold & Freedman, 1965; Prehn, 1967). In studies specifically involving H-2 antisera produced against normal tissues, Pizarro, Hoecker, Rubinstein & Ramos (1961) failed to demonstrate these antigens in newborn tissues, but Schlesinger (1964), using a different technique, did describe the absorption of H-2 agglutinins by $10\frac{1}{2}$-day fetuses. Naked cleaving mouse eggs from the 8-cell to blastocyst stage were killed when cultured *in vitro* with allogeneic cytotoxic antibody, but were unaffected when cultured with antiserum directed specifically to H-2 antigens (Heyner *et al.*, 1969). These authors suggest that non-H-2 transplantation antigens are present on the pre-blastocyst egg in the mouse.

There have been suggestions that embryonic tissues might express embryo-specific and transient developmental (phase-specific) antigenicity. Experiments in several mammals have indicated that, in the tissues of developing embryos, antigenic factors are present which are characteristic of a particular period of morphogenesis and absent from the adult animal (see review by Volkova & Maisky, 1969). Kirby (1969a) has suggested that successive blastocyst implants from allogeneic or syngeneic donors in extra-uterine sites meet second-set rejection. Further investigation of this possibility is needed.

While there is evidence that early developmental stages are antigenic, the time of the first appearance of paternal transplantation antigens in the embryo has not yet been made clear. Preliminary observations have suggested that the culture *in vitro* of 8-cell zona-free C57BL × CBA F_1 and C57 inbred eggs in anti-CBA serum does not affect their viability or their capacity to form blastocysts and trophoblastic outgrowths. CBA inbred eggs, however, show reduced viability under these conditions (Clarke, unpublished observation). If these results are confirmed they would suggest that oviductal hybrid embryos are not effectively expressing paternal antigens.

Recent studies on the appearance of enzymic products of paternal alleles in the mouse have shown that by the 6th day of pregnancy the hybrid pattern of glucose phosphate isomerase-1 is discernible (Chapman, Whitten & Ruddle, 1971). This locus is, therefore, transcribed and translated before the 5th day, and consequently it may be concluded that at least part of the paternal contribution to the genome of the egg is active at this early stage.

Before implantation, the egg is enveloped in the zona pellucida and several investigators have suggested that this membrane might limit immunological contact between the maternal system and the unimplanted egg, masking the presence of antigens on the blastocyst (Simmons & Russell, 1966, 1967a; Shelesnyak, Marcus, Kraicer & Lobel, 1967; James, 1969; Heyner *et al.*, 1969). However, in lactational delay, the zona is shed at about the normal time but implantation is successful 4 days later. In ovariectomized, progesterone-maintained pregnant females, highly immunized to paternal antigens, no effect on blastocyst viability was observed after 10 days of zona-free existence

(Kirby, 1969b, 1970). The presence of the zona pellucida is, therefore, not essential for embryonic viability and development.

Maternal/fetal exchange

The fetus undoubtedly expresses the antigenic determinants which are the first necessity for a homograft reaction. The second necessity is physical contact between the cells of the fetus and its mother.

Trophoblastic cells of fetal origin gain access to the maternal system in considerable numbers. Syncytial embryonic trophoblast can be detected in the blood vessels of the uterus of most normal pregnancies in a variety of animals (see Billington, 1970).

Evidence that embryonic cells other than trophoblastic cells cross the placenta in normal pregnancies is limited. Fetal blood cells gain entry to the human mother through breaks in the blood vessels of the placenta and can be demonstrated in the maternal blood (Lee & Vasquez, 1962). In all likelihood, if red blood cells pass from fetus to mother, then some fetal lymphocytes will also do so.

The majority of reports imply that the passage of cells from mother to fetus in normal pregnancies is a rare occurrence. In humans, despite extensive studies of male neonates and abortuses, maternal cells have been found in only four instances (Taylor & Polani, 1965; Kodawaki, Thompson, Zuelzer, Woolley, Brough & Gruber, 1965; Lischner, Punnett & di George, 1967; Walknowska, Conte & Grumbach, 1969).

Human maternal cancer cells have occasionally become established in newborn infants (Retik, Sabesin, Hume, Malmgren & Ketcham, 1962) but leukaemic cells do not apparently reach the fetal circulation in mice (Loewenstein, Hughes, Hofer & Ketchel, 1971).

A report of the passage of large numbers of maternal cells into the mouse fetus (Tuffrey, Bishun & Barnes, 1969) has not been confirmed using similar techniques (Billington and his colleagues, 1969) or by a different experimental approach (Seller, 1970). It would seem, therefore, that the placental barrier as postulated by Medawar (1953) is effective in preventing the interchange of most maternal and fetal cells.

Although this barrier is effective in preventing cellular exchange, it appears to act as a selectively permeable barrier to immunoglobulins (see Brambell, 1966, and Wild, 1966). Placental passage is apparently controlled by the nature of the heavy chain. In some mammals IgG molecules readily cross the placenta whereas IgA molecules, which are similar in size, and IgM molecules, which are larger, do not. In other mammals, no antibodies cross the placenta, but there is selective passage across the yolk sac (see Brambell, 1966). Following incompatible blood-group matings in man, transplacental passage of maternal isoantibodies may result in haemolytic disease of the newborn. However, Lanman & Herod (1965) have shown that, in the rabbit, humoral antibodies can pass through the placenta and home on specific fetal target organs without causing visible harm.

THE NATURE OF THE TROPHOBLASTIC BARRIER

Medawar (1953) first suggested that the presence of a physical barrier between the fetus and the mother might be responsible for fetal survival from the homograft reaction. Anatomically, the placenta, which separates maternal and fetal blood, could provide such a barrier. The only component of the conceptus which comes into direct contact with maternal tissue is the trophoblast, and this part of the placenta has been shown to persist throughout pregnancy (Bradbury, Billington & Kirby, 1965).

The essential rôle of the trophoblast in the immunological problem of pregnancy has been shown by transplantation studies. Trophoblastic growths and blastocysts transplanted to extra-uterine sites develop successfully without rejection in mice (Kirby, 1960, 1965; Simmons & Russell, 1962; McLaren & Tarkowski, 1963), in rats (Kirby, 1962), in guinea-pigs (Bland & Donovan, 1965) and in hamsters (Billington, 1966). Even xenogeneic grafts grow and proliferate successfully: mouse in rat (Kirby, 1962; Simmons & Russell, 1967b) and mouse in hamster (Billington, 1966).

Trophoblastic grafts grow in extra-uterine sites in the presence of a pre-existing immunity in mice (Kirby *et al.*, 1966; Simmons & Russell, 1967a; Clarke, 1971) while embryonic implants in the same situation are destroyed (Simmons & Russell, 1967a; Kirby *et al.*, 1966).

The failure of the trophoblast to express strong histocompatibility antigens has been demonstrated serologically in mice, using haemagglutination inhibition techniques and in human trophoblast using immune absorption techniques (Schlesinger, 1964; Seigler & Metzgar, 1970).

Although trophoblast does not express histocompatibility antigens in the normal manner, there is some evidence that it is not entirely devoid of all antigenicity. Simmons & Russell (1967b) have demonstrated species-specific trophoblastic antigens in the rat and mouse. The possession of tissue-specific antigens has also been suspected from reports that antibodies to trophoblast appear in the *post-partum* period in normal human pregnancies and in the serum of women after abortion (Hulka, Brinton, Schaaf & Baney, 1963). Anti-trophoblastic antibodies cannot be detected during a normal pregnancy and may only occur after normal or abnormal separation of the placenta.

The mechanism by which the trophoblast fails to express completely the antigens expressed by the rest of the fetal tissue is disputed. Two main hypotheses have been put forward.

In 1964, Kirby, Billington, Bradbury & Goldstein postulated that transplantation antigens are present on trophoblastic tissue but their expression is prevented by an extracellular layer which they called the fibrinoid layer. Simmons & Russell (1966), in contrast, believe that trophoblastic cells fail to develop histocompatibility antigens.

Conflicting experimental evidence as to the nature and properties of the fibrinoid layer has led to disagreement about the existence and capacity of an extracellular layer to mask trophoblastic antigenicity. Histochemical and electronmicroscopic observations of trophoblastic tissue supported the existence

of a 'masked' antigenicity. The physical presence of a darkly staining surface coat of mucoprotein rich in sialic and neuraminic acids was identified around murine trophoblast cells and seemed to form a boundary layer between maternal and fetal tissue (Bradbury *et al.*, 1965). The first appearance of this extracellular layer was found to coincide with the time at which an embryonic implant did not succumb to a pre-existing immunity when transplanted to an extra-uterine site (Potts, 1965). The presence of this extracellular layer has subsequently been identified in the placenta of many mammals, including man (Wynn, 1967; Boyd, Hamilton & Boyd, 1968; Bradbury, Billington, Kirby & Williams, 1969). However, other workers have failed to detect the presence of an extracellular layer round murine trophoblastic cells (Simmons, Cruse & McKay, 1967), it does not appear to be present on rabbit trophoblast (Tai & Halacz, 1967) and, in the rat, where fibrinoid is present, it has been reported not to form an intact barrier between trophoblast and decidua (Martinek, 1970).

Experimental evidence supporting the presence of a barrier surface coat surrounding trophoblast cells has come from the observations of Currie & Bagshawe (1967). They showed that, when choriocarcinoma cells (malignant trophoblast) were treated with trypsin and cultivated with allogeneic lymphocytes *in vitro*, they died, whereas syngeneic tissue survived. This experiment is open to criticism as choriocarcinoma cells were not tested with syngeneic lymphocytes as a control.

Currie and Bagshawe have suggested a possible mechanism by which the fibrinoid barrier could function, from a consideration of the behaviour of cancer cells which appear to be surrounded by a similar substance. They postulate that tumour antigens and trophoblastic antigens escape recognition in the same way, and believe that sensitized lymphocytes, which are known to be negatively charged, could be electrostatically repulsed if a similar negative charge was present on the extracellular layer. Stoward (1968) added some support for the presence of a negative charge on the fibrinoid layer by showing that a high degree of sulphation occurred in the mucoprotein component of fibrinoid material. Sulphation is known to be associated with negatively charged cells, but the possession such of electro-chemical properties by the sialomucins of the extracellular layer has been disputed by Good (1967), who believes that electrostatic repulsion in an aqueous medium would not be possible without damaging the tissues.

Currie, van Doorninch & Bagshawe (1968) demonstrated that neuraminidase-treated mouse trophoblast was capable of inducing immunity when transplanted to allogeneic recipients, whereas untreated trophoblastic tissue did not have this property. These authors suggested that neuraminidase enzymatically digests extracellular fibrinoid and unmasks the transplantation antigens present on trophoblastic cells.

This result has not been confirmed by Simmons and his colleagues, who failed to elicit any degree of skin-graft immunity when mouse trophoblast was incubated with neuraminidase and transplanted into allogeneic hosts. In addition, incubation of trophoblast with neuraminidase also failed to interfere with its subsequent proliferation in extra-uterine sites of highly immunized allogeneic hosts (Simmons, Lipschultz, Rios & Ray, 1971).

There is some indication that the action of neuraminidase may render cells generally more immunogenic. Mice receiving allogeneic lymphoid cells treated with neuraminidase reject allogeneic skin grafts faster than if inactivated neuraminidase is used. This suggests that the action of neuraminidase does not unmask specific antigenic determinants on trophoblastic cells, but non-specifically renders them more susceptible to immunological processing by recipients (Simmons, Rios & Ray, 1971). This is in agreement with earlier findings that neuraminidase increases the immunogenicity of a variety of cells (Simmons, Rios & Ray, 1970).

IMPLANTATION

While the placenta may, in some way, form a barrier which prevents the immunological rejection of the fetus, some other mechanism must be involved during implantation and before the formation of the definitive placenta. The actual process of implantation involves a complex series of interactions between endometrium and blastocyst, the nature and control of which are poorly understood. This phase of the reproductive cycle is, nevertheless, of great importance, for it is during this period that the highest mortality of embryos occurs (Hertig, Rock, Adams & Menkin, 1957; Brambell, 1948; Adams, 1955).

There are a number of observations which have led to the suggestion that immunological reaction could occur between mother and fetus at the time of implantation. In the rabbit, accumulations of lymphocytes occur in the sub-epithelial space at the site of implantation (Potts, 1965), and antigens present in the blastocoelic fluid have been recovered from the uterine stroma and lumen (Beier, 1968). In the rat, uterine cells transfer substances into the sub-epithelial space at the site of implantation (Vokaer, 1952). These observations indicate that the passage of substances between blastocyst and uterus is possibly quite frequent.

One of the early signs of implantation in many mammals is the development of decidual tissue in the uterine stroma in the region of the blastocyst. When pre-trophoblastic stages of development are transplanted to ectopic sites they are rejected if the host has been sensitized to the transplantation antigens of the donor. When transferred to the uteri of similarly prepared hosts, in the presence of decidual tissue, rejection does not occur (Kirby *et al.*, 1966). This observation led to the suggestion that decidual tissue may provide protection from immunological consequences of maternal/fetal incompatibility during this early stage of development. The nature of the protection is unclear, as decidual tissue is well supplied with blood and lymphatic vessels. The presence of tight junctions between cells in decidual tissue, reported by Potts (1968), may be relevant in limiting cellular contact and passage of cells between the mother and conceptus at this stage.

There is only meagre evidence that immunological interactions around the time of implantation significantly affect development. From measurements of the amount of decidual tissue in mice on the 7th day of pregnancy Hetherington (1970) has postulated that during the pre-implantation phase of pregnancy an immunological reaction occurs which affects either the blastocyst's ability to

induce the decidual cell reaction or the mother's ability to respond. Variations in the amount of decidual tissue were not due to differences in the rate of development of the pre-implantation blastocyst, which is known to depend on both the blastocyst genotype and the maternal environment (Whitten & Dagg, 1961; Gates, Doyle & Noyes, 1961; McLaren, 1968).

This observation induced an investigation into the effects of maternal pre-immunization to paternal antigens on the decidual cell reaction. A significant reduction in the amount of decidual tissue was found in pre-immunized females. This reduction was due to a specific reaction to paternal antigens and did not occur when females were immunized to xenogeneic antigens (Clarke & Hetherington, 1971).

Decidual tissue may protect the developing conceptus (Kirby *et al.*, 1966), provide nutrition, or contain the invasiveness of trophoblast (Kirby, 1965). Possible effects on the extent of growth of this cellular reaction, which can apparently be brought about by immunological phenomena, might affect either placental or fetal weight in later pregnancy. In no instance, however, where decidual weight is apparently affected by these reactions, is there an effect on placental weight. There is, however, an indication that the decidual response on the 7th day and fetal weight on the 18th day of pregnancy are positively correlated (Hetherington, 1970; Clarke & Hetherington, 1971).

Whether the effect on fetal weight is the direct consequence of changes in the decidual response or whether both fetal and decidual weights are dependent on a third factor is not known.

UNRESPONSIVENESS OF THE MATERNAL IMMUNOLOGICAL SYSTEM

Investigations into the immunological state of the mother during and after pregnancy have demonstrated that, although immunologically competent, she shows a degree of partial unresponsiveness to fetal and paternal skin grafts (Woodruff, 1957; Breyere & Barratt, 1960). A similar state of partial unresponsiveness has been found to occur following immunization against certain tumours. In these circumstances, tumour graft survival appears to be increased by immunization, and the phenomenon has been termed immunological enhancement (Kaliss, 1957, 1958; Moller, 1963b, 1965; Hellström & Hellström, 1969).

The parallels between the two phenomena have been emphasized by Kaliss & Dagg (1964). Immunological enhancement of tumour homografts is apparently mediated by circulating 'barrier' antibodies in the presence of immune lymphocytes (Kaliss, 1957), while fetal survival also occurs in the presence of antifetal antibodies (Lanman & Herod, 1965) and immune lymphocytes (Soren, 1967). Tumour enhancement only occurs with tumours which differ from the host with respect to the H-2 locus. These tumours have surface antigens in a low concentration (Moller, 1963b) and are analagous to fetal trophoblast, which is similarly weakly antigenic.

Although immunological enhancement has only been adequately demonstrated in response to tumour proliferation, experimental evidence is available to

suggest that the phenomena might occur in the fetal–maternal relationship. In the presence of circulating 'barrier' antibodies, any exposed antigenic sites on trophoblast cells may become coated by these antibodies and thereby protected from attack by sensitized maternal lymphocytes.

Enhancing antibodies produced in response to the presence of a tumour in an allogeneic host can be passively transferred by iso-antisera (Kaliss, 1957, 1958; Gorer, 1958). This property of passive transfer distinguishes enhancement from conventional tolerance.

There is no direct or conclusive evidence that maternal unresponsiveness to paternal skin grafts can be passively transferred. Initial attempts to demonstrate such a transfer, using serum from multiparous females, were not successful (Kaliss & Dagg, 1964). Pregnancy haemagglutinins, however, have a short half-life on transfer (Rubinstein & Kaliss, 1964) and, in a similar study with more extensive immunization, Currie (1969) claims to have passively transferred enhancement to allogeneic tumours and skin grafts by the injection of allogeneic pregnant serum.

Serum taken from pregnant mice has been shown to be capable of protecting fetal cells *in vitro* from attack by immune lymphocytes (Hellström, Hellström & Brawn, 1969). These workers have shown that lymphocytes from mice carrying antigenically foreign fetuses inhibit the growth of fetal cells of the same genetic type. When serum from the pregnant mice was added to the cultures the cells remain viable. The authors point out that it cannot be concluded that the protective serum factor is an immunoglobulin. However, in tumour systems, a similarly acting factor, which results in protection from immune lymphocytes, was demonstrated to be a 7S immunoglobulin (Hellström, Hellström & Pierce, 1968; Hellström & Hellström, 1969).

Evaluation of the mechanisms of maternal host unreactivity in pregnancy has involved attempts to break the 'enhancement' and leave the embryo relatively unprotected. Avery & Hunt (1968) have shown that the nature of the maternal response to a *post-partum* skin graft after an inter-strain pregnancy may depend on the route of administration and dose of the immunizing stimulus given during pregnancy. Thus, the low doses of antigens received by the mother from the fetal circulation might induce enhancement while the larger doses of antigens given in pre-immunization experiments might result in sensitization. Halpern and his colleagues (1963) found that non-specific stimulants of cellular immunity overcame the enhancement of tumours, and Currie (1969) found that fetal reabsorptions increased in inter-strain pregnancies given several injections of the adjuvant *Corynebacterium parvum*. Currie (1969), however, using large doses of paternal spleen cells, found that immunization did not affect litter size or the number of implantation sites. When doses of paternal tumour cells were used for pre-immunization, fetal death rate tended to be increased.

The kinetics of immunological enhancement are uncertain and many of these attempts to overcome specific unreactivity in pregnancy may have used inappropriate conditions. However, it is clear that the presence of enhancement must depend on some humoral balance between enhancing antibodies and the cytotoxic antibodies involved with the immune lymphocytes in sensitization.

The rôle of the spleen has been shown to be critical in the induction of tumour enhancement (Old, Clarke, Benacerref & Stockert, 1962). Enhancement does not occur after splenectomy in mice (Ferrer, 1968a; Prehn, 1959; Moller, 1965), and sera from splenectomized mice does not result in enhancement on passive transfer (Ferrer, 1968b).

A preliminary experiment (Clarke & McLaren, unpublished) has suggested that splenectomy in the presence of immunity to the paternal strain antigens does not affect fetal or placental weight but may increase the rate of fetal resorption in inter-strain pregnancies.

Some doubt as to the importance of maternal unresponsiveness during pregnancy has been provided by the work of Currie (1970). He found that the development of male strain fibrosarcoma in pregnant mice previously immunized to paternal antigens was unaffected by a concurrent pregnancy. This suggested that pregnancy did not significantly affect the immune state of cell-mediated immunity to paternal tissue.

DISCUSSION

The two protective mechanisms that have received the most attention have been: (1) the presence of anatomical barriers separating maternal and fetal cells, and (2) the participation of 'enhancing' or 'barrier' antibodies at the trophoblastic interface.

The existence of physical barriers between the mother and embryo, namely the trophoblast and possibly also, earlier in pregnancy, the zona pellucida and the decidual cell reaction, is thought to limit maternal–fetal antigenic exchange and to minimize the consequences of maternal cellular mechanisms.

Because these barriers are incomplete, the circulation of the mammalian mother is subjected to small doses of fetal antigens. This antigen stimulation causes the formation of conventional anti-fetal antibodies and sensitized lymphocytes. In addition, it may induce 'barrier' antibodies. These latter antibodies may protect the fetus by attaching to trophoblast and preventing sensitized maternal lymphocytes from damaging fetal cells. The trophoblastic barrier thus serves the dual function of limiting antigenic contact and acting as a target site for the attachment of enhancing antibody. The incomplete nature of the barrier and the weak antigenicity of trophoblast cells could well be essential for the production of this type of immunity. The rôle of barriers and enhancing antibodies have been considered in greater detail above (see p. 106 and p. 109).

We might postulate that 'barrier' antibodies have been evolved to meet the immunological problems of viviparity. There seems to be no obvious selective advantage in their demonstrated action on tumour growth. This hypothesis would be disproved if enhancing antibody were demonstrated in animals that are not viviparous.

Although the fetus appears to be isolated from the effects of maternal cellular immunity it is not entirely isolated from the humoral components of the maternal immunological response. Other protective mechanisms must be present.

The placenta has been shown to act as a selective barrier to the passage of maternal immunoglobulins. It is known that only IgG 7S immunoglobulins are readily transferred across the placenta. IgG 19S, IgA, IgM and IgE are not transmitted (Cann, Brown, Gajdusek, Kirkwood & Sturgeon, 1951; Gitlin & Morphis, 1969). It should be noted that, in animals where transmission is by the yolk sac, there is no evidence of selectivity (see Brambell, 1970). However, in general, the yolk sac route is of less importance than later colostral transmission (Brambell & Halliday, 1956).

The state of immunological development of the embryo itself provides some possible clues about how embryonic cells could escape damage from IgG 7S antibodies. On the one hand, the fetus appears capable of humoral immunoglobulin synthesis (see Adinolfi & Wood, 1969; Solomon, 1971) and it possesses competent cellular immunity as shown by its capacity to reject skin grafts *in utero* (Silverstein & Lukes, 1962). On the other hand, there have been reports of fetal inability to produce some components of complement and of the inefficiency of fetal phagocytes.

IgG 7S immunoglobulin consist of two major components, cytotoxic antibody and cytophilic antibody, both of which need the participation of other factors to cause cell damage.

Cytotoxic antibody requires complement to lyse cells. It appears that although mammalian fetuses produce some complement, the levels are relatively much lower than in adults and the production of some components is deficient until late in fetal life. The concentration of complement in newborn humans is half that in the mother (Fireman, Zuckowski & Taylor, 1969; Gewurz, Pickering & Good, 1968). Fetal and newborn lambs have no complement in early pregnancy and very little until after birth (Rice & Silverstein, 1964). Complement is present in fetal pigs at 40 days, but the level is much lower than that in the adult, and component C_4 is extremely low until birth (Day, Pickering, Gewurz & Good, 1969).

In mammals, the several components of complement in the presence of specific antibody act sequentially to produce cell lysis. Only one of the components need be absent, or present in inadequate amounts, for the complement system to be ineffective. Lack of an effective system could protect the embryo from maternal anti-fetal cytotoxic antibody.

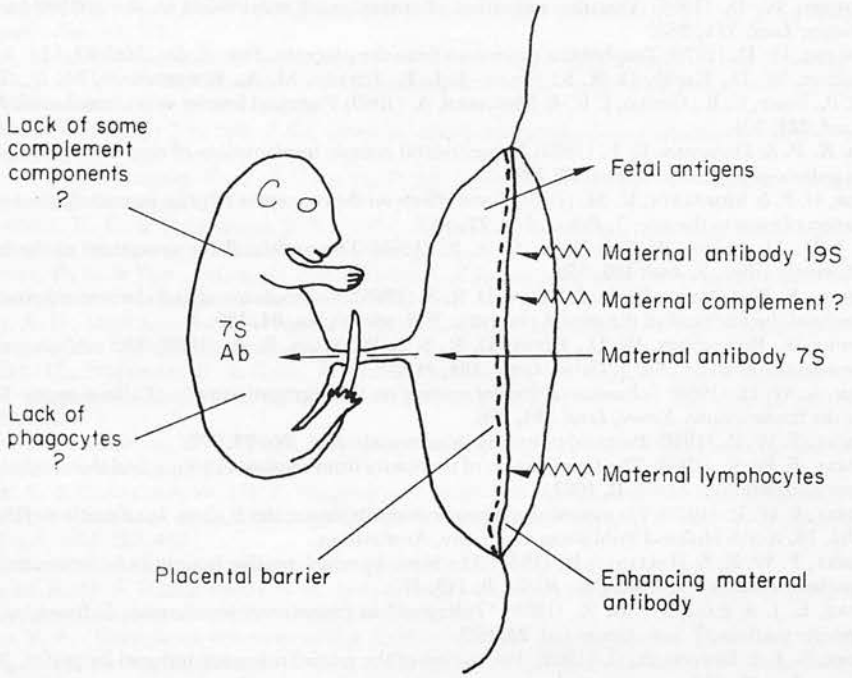
If protection operates in this way, it is necessary that maternal transfer of complement to the fetus does not occur. In the few cases in which it has been studied, the placenta has been found to be impermeable to complement (Tachibana & Rosenberg, 1966).

Cytophilic antibody can only kill cells with the help of the phagocytes. The antibody acts by attaching to specific foreign cells and the complex is then engulfed by the phagocyte and destroyed. In fetal rats, Reade & Jenkin (1964) have found that although phagocytes are present and are capable of engulfing foreign particles coated with appropriate antibody, they have no bactericidal capacity, a property readily manifested by adult phagocytes. The phagocytic activity of leucocytes from premature babies has been shown to be very inefficient *in vivo* (Gluck & Silverman, 1957).

We therefore suggest that the apparent lack of complement and the in-

efficiency of phagocytes in the fetal system could be factors protecting the embryo from anti-fetal IgG 7S antibody.

Text-figure 1 is an attempt to summarize fetal protection. If it is representative of the truth we must conclude that the development of viviparity has demanded the evolution of several protecting systems. Among them are the presence of the



TEXT-FIG. 1. Possible mechanisms for the protection of the mammalian fetus against the homograft reaction. In some mammals antibody transmission occurs by way of the yolk sac.

trophoblastic barrier, the semi-permeability of the placenta, the induction of enhancing 'barrier' antibody and, possibly, the retarded development of some subsidiary immunological capacities of the fetus.

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