STUDIES ON THE IMMUNOBIOLOGY OF MOUSE FETAL MEMBRANES: THE EFFECT OF CELL-MEDIATED IMMUNITY ON YOLK SAC CELLS IN VITRO

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Summary. The susceptibility of the mouse yolk sac to immune cell lysis has been investigated using an in-vitro microcytotoxicity test. Monolayer cultures derived from preparations of whole yolk sac and of the outer endodermal component were used as target cells. With combinations of mice differing either at major (H-2) or minor (non-H-2) histocompatibility loci, both types of culture showed extensive destruction following incubation with spleen cells from specifically immunized donors. Possible reasons for the survival of the yolk sac in vivo, despite the demonstration of antigenic determinants recognizable in vitro, are discussed, together with the significance of these findings for understanding of human fetal membrane function.

INTRODUCTION

It has long been recognized that the mammalian conceptus occupies a privileged position as a successful allograft within the uterus of a genetically dissimilar mother. Several hypotheses have been advanced to explain the apparent lack of maternal immunological rejection reactions, and it is now clear that a number of factors are likely to be involved (see reviews by Beer & Billingham, 1971; Billington, 1971). It has generally been considered, however, that the placenta, and in particular the trophoblast component, is the only region of importance in the feto-maternal immunological relationship. The fetal membranes, which also lie at the boundary of maternal tissues, have been almost completely neglected from this point of view. Although the precise structure and anatomical arrangements may vary from species to species, there is always extensive contact between the outermost fetal membrane and the uterine tissues. Over this entire portion of the non-placental surface, the conceptus should therefore be potentially vulnerable to any maternal immunity.

In the mouse, as in many other rodent species, the fetal membrane in this position is the yolk sac. This differentiates very early in development and has a diverse range of physiological functions. Although it is initially surrounded by
the acellular Reichert's membrane and by a layer of trophoblast giant-cells, these break down in the last half of pregnancy and expose the yolk sac to the uterine lumen (Text-fig. 1). Since this fetal membrane survives not only in normal pregnancy but also when the female has been presensitized to paternal strain antigens, it must in some way avoid or resist immune attack. An important point to establish in this respect is the antigenic status of the yolk sac. We have therefore carried out a series of studies to investigate the reaction of this fetal membrane to both humoral and cell-mediated immunity. In this communication, we report on the susceptibility of in-vitro preparations of the membrane to immune cell lysis, in systems involving both major and minor histocompatibility antigen differences between effector and target cells.

![Text-fig. 1. Final arrangement of fetal membranes in the mouse. The yolk sac is directly exposed to the uterine lumen following the breakdown of Reichert's membrane and the trophoblast giant-cell layer between Days 13 and 14 of pregnancy.](image)

**MATERIALS AND METHODS**

*Animals*

Two combinations of inbred strains of mice were used. Material was obtained either from the C57BL(H-2^b^) and A(H-2^a^) strains, which differ at the major histocompatibility (H-2) locus (as well as at other minor loci), or from the CBA/Ca(H-2^k^) and C3H/He-mg(H-2^k^) strains, which differ only at minor (non-H-2) loci.
**Immunization schedule**

The effector cells used in the test system were spleen cells from normal adult animals (controls) and from animals immunized against the antigens of the target cell donors. Adult males of the C57BL strain were immunized by a full-thickness skin graft followed after 2 weeks by weekly intraperitoneal injections for 2 to 6 weeks of $5 \times 10^7$ washed spleen cells from male A-strain donors. Mice of the C3H/He-mg strain were similarly immunized using CBA/Ca tissues. Immune effector cells were taken 6 to 10 days after the final injection.

**Preparation of yolk sac target cells**

The mouse yolk sac is essentially a bilaminar membrane which, due to inversion of the germ layers during development, is composed of an outer layer of endoderm cells and inner mesodermal elements (Pl. 1, Fig. 1). Target cells were obtained both from the entire membrane and from the endodermal layer from donor mice at a number of different stages of timed pregnancies before and after the breakdown of Reichert's membrane.

**Whole yolk sac preparations.** Donor mice at 10, 13 and 14 days post coitum (p.c.) were killed by cervical dislocation and the uterine horns were exposed. The uterine wall was slit along its length using fine scissors and each conceptus was teased out with its placenta attached. All subsequent operations were carried out under sterile phosphate-buffered saline (PBS) at room temperature. Before 14 days p.c., the conceptuses are covered with a layer of trophoblast giant-cells and Reichert’s membrane. These were peeled away with watchmakers forceps,
care being taken not to puncture the underlying fetal membranes. The yolk sac was then cut around the edge of the placenta, the fetus was removed, and the amnion was dissected away. The entire membrane was chopped into small pieces, washed twice in PBS to reduce contamination with erythrocytes, and incubated in a centrifuge tube in 0.25% trypsin solution at 37°C for 30 min. The resulting cell suspension was filtered through stainless steel gauze to remove débris and spun at 165 g for 10 min. The supernatant trypsin was discarded, the remaining cell pellet was resuspended in 1 ml of complete medium (RPMI (Biocult) plus 10% fetal calf serum), and the cells were counted and adjusted to the desired concentration.

Pure endoderm preparation. Entire conceptuses at 14 and 16 days p.c. were isolated from the uterus as described above. A loop of sterile suture thread (Arbrasilk gauge 5/0) was placed with forceps over the placenta, manoeuvred into position, and tightened to compress the margins of the yolk sac and ligate the umbilical cord at its insertion into the placenta. With a fine scalpel blade, the membranes and cord were cut above the ligature to remove the placenta intact and leave a closed yolk sac encapsulating the fetus. This was suspended by the thread and immersed in a 0.5% trypsin solution. Four to six such preparations were obtained and placed in the same vessel at 37°C for 30 min. The method is illustrated in Text-fig. 2. At the end of the incubation period the sacs were removed to a Petri dish and fresh trypsin was pipetted gently over them. The washings were pooled with the supernatant from the vessel and the cells were spun down, washed and treated as described in the previous section. This procedure removes endodermal cells from the yolk sac without damaging the basement membrane and introducing contaminating mesodermal elements.

In-vitro microcytotoxicity test

The method used to examine the susceptibility of the yolk sac target cells to immune lysis was a modification of the microcytotoxicity test described by Takasugi & Klein (1970). This depends upon the recognition of antigenic determinants on the surface of the target cells by effector spleen cells obtained from donor mice immunized against tissues bearing such determinants. The effect on the target cells is a complex multi-stage phenomenon resulting in cell death and detachment from the surface of the culture vessel.

In the first series of experiments, whole yolk sac cells were seeded out into Cooke microtitre plates at a concentration of $10^4$ cells/well in the complete medium previously described. After incubation for 24 hr under 5% CO₂ in air, the cells formed monolayer growths on the bottom of the wells. In a
second series, monolayers of pure endodermal cells were obtained in a similar manner. Control cultures of embryonic fibroblasts of the same age and at the same concentration were also set up with each experimental group.

All cultures were then incubated for 48 hr with either $5 \times 10^5$ or $10^6$ spleen cells/well, giving an effector:target cell ratio of 50:1 or 100:1. Immune allogeneic and control non-immune allogeneic spleen cell cultures were always set up in parallel. In addition, the specificity of the immune cell killing was tested using syngeneic target monolayers of both fibroblasts and yolk sac cells.

All experiments were initially carried out using the two strains of mice differing at the major histocompatibility antigen complex. A smaller group, using only the whole yolk sac preparations as target monolayers, was investigated with the weaker non-H-2 combination.

The assessment of target cell death was made by washing the cultures twice in PBS to remove cell débris and excess spleen cells, fixing in methanol for 30 min, staining with freshly prepared Giemsa, and examining under the binocular microscope for the presence of stained surviving cells.

RESULTS

Test system involving major histocompatibility antigen differences

At both effector cell levels, all A-strain yolk sac target monolayers were extensively destroyed (>90%) by immune allogeneic C57BL spleen cells, irrespective of the day of pregnancy on which they were originally obtained. The degree of destruction was comparable to that seen with the control embryonic fibroblast monolayers. Non-immune allogeneic cells had no observable effect on yolk sac cells (Pl. 1, Fig. 2) or fibroblasts. The possibility of non-specific killing by immune cells at the levels used was excluded by the

<table>
<thead>
<tr>
<th>Table 1. Effect of immune (+) and non-immune (−) C57BL(H-2b) spleen cells on H-2 incompatible A-strain (H-2a) mouse fetal tissues in vitro</th>
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</thead>
<tbody>
<tr>
<td><strong>Target cells</strong></td>
</tr>
<tr>
<td>Yolk sac</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Pure endoderm</td>
</tr>
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<td>Fibroblasts</td>
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* Expressed as number of culture wells showing >90% target cell destruction.
lack of effect on C57BL monolayers of immune C57BL anti-A spleen cells. This specificity control was carried out on a total of thirty wells of fibroblasts and ten wells of whole yolk sac preparations.

Since the yolk sac cultures consisted of a mixed population of cells, presumably derived from all elements of the membrane (see Pl. 1, Fig. 2), it was not possible to assess the involvement of any one cell type. The susceptibility of the important outer endodermal cell layer was therefore investigated. The typical appearance of cultures of this cell type is shown in Pl. 1, Fig. 3, and the uniform morphology may be contrasted with that seen in the mixed cultures. There was no difference in the susceptibility of the pure endoderm to immune cell attack, and all monolayers of A-strain origin showed extensive destruction.

The stages of pregnancy examined and the number of experiments carried out in these series are detailed in Table 1.

Test system involving minor (non-H-2) histocompatibility antigen differences

Cultures of whole yolk sac obtained from CBA/Ca conceptuses at 14 days of pregnancy and control 14-day embryonic fibroblasts all showed significant destruction (>60% of the monolayer) following incubation with spleen cells from immunized C3H/He-mg donors. Non-immune C3H/He-mg spleen cells had no observable effect on similar cultures. The results are shown in Table 2.

<table>
<thead>
<tr>
<th>Target cells</th>
<th>Gestational age (days)</th>
<th>Effector cells</th>
<th>Cytotoxic effect (&gt;60%)</th>
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<tbody>
<tr>
<td>Yolk sac</td>
<td>14</td>
<td>+</td>
<td>19/19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>0/19</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>14</td>
<td>+</td>
<td>12/12</td>
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<tr>
<td></td>
<td></td>
<td>-</td>
<td>0/12</td>
</tr>
</tbody>
</table>

+, immune spleen cells; -, non-immune spleen cells.

DISCUSSION

One of the theories proposed to explain the survival of the conceptus as an allograft is that the uterus may share with such sites as the anterior chamber of the eye and the brain the property of immunological privilege (Billingham, 1964). Although there are certain reservations (Billington, 1967), there is evidence that this is not a major factor, since animals may be immunized by way of the intratuterine route and reactions of transplantation immunity can take place within this environment (Beer & Billingham, 1971). The survival of the fetal membranes is therefore of some interest. It is clear from the present studies that the yolk sac as a whole, and in particular its outer endodermal component, possesses cell-surface antigenic determinants in common with non-

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exposed embryonic tissues (fibroblasts) and recognizable by spleen cells from specifically immunized donors. Although the classification of the determinants recognized by immune lymphocytes is not yet fully established, the available evidence indicates that these are either identical to or closely associated with the serologically defined (SD) antigens of the major histocompatibility (H-2) complex (Henney, 1973). The killing of CBA/Ca target cells by immune C3H/He-mg spleen cells suggests that antigenic determinants of the minor histocompatibility loci (non-H-2) can also be recognized. Whatever the precise nature of the antigens involved, their expression on the fetal membrane renders it susceptible to immune cell lysis in vitro.

There are very few previous reports on the immunological properties of fetal membranes, and all are based upon transplantation to ectopic sites in vivo. In the earliest of these, it was recorded that mouse “chorionic membranes” survived for 13 days on a “denuded surface upon the dorsum of a mouse” (Douglas, Conway, Stark, Joslin & Nieto-Cano, 1954). Although the stage of pregnancy of the donor mice was not given, and the nature of the membranes was not defined, the rejection time of the grafts on “mice of widely divergent genetic strains” suggests that they possessed significant antigenic strength. This approach was also used by Avery & Hunt (1967), who transplanted pieces of yolk sac to prepared sites on the thorax of allogeneic recipient mice. They concluded that transplantation antigens were present on this membrane from at least 14 days of gestation. Since, however, they observed a less vigorous rejection of the membrane when the outer (endodermal) layer was in apposition to the graft bed, they commented that “The question not adequately resolved is whether the antigenicity of the yolk sac is a function of all its cellular elements, or whether only the elements derived from extraembryonic mesoderm are antigenic.” Our present findings may be taken as lending support to their assertion that the yolk sac is antigenic, but clearly indicate that the endoderm cells do not differ in this respect.

Two other studies bearing on this problem utilized the peritoneal cavity as a recipient site for yolk sac transplantation. Payne & Payne (1961) claimed that, in contrast to adult skin fragments, rat yolk sacs taken on the 14th or 15th day of pregnancy survived, and differentiated into a variety of tissues, when transplanted into the omentum of outbred host rats. It is difficult to reconcile these findings with the later report of Avery & Hunt (1968) which showed that, under similar conditions in allogeneic hosts, the mouse yolk sac elicited a cellular response leading to its rejection. Even in isogeneic hosts, the membranes did not give rise to highly differentiated secondary tissues. Except for the unlikely possibility of species difference, a reasonable explanation for this discrepancy would be that the rat yolk sac fragments contained contaminating embryonic tissue, which is well known to undergo a teratoma-like development following ectopic transfer.

Since the mouse yolk sac as a whole is undoubtedly antigenic, and our in-vitro approach indicates that the outer endodermal cell layer possesses recognizable antigenic determinants, some explanation must be sought for the apparent lack of susceptibility of the membrane to maternal immune attack. Firstly, although antigens can be identified in vitro, where target cell orientation
may be random, it is possible that their distribution in vivo could be polarized in such a way that they would not be exposed on the outer surface of the endoderm cells. Secondly, as suggested in the case of trophoblast (Kirby, Billington, Bradbury & Goldstein, 1964), antigens may normally be present but masked by some coating material that is removed by trypsinization of the cells during preparation for culture. With regard to the situation in vivo, an important point to recognize is that the membrane is not vascularized by host (maternal) blood vessels in the manner of a surgically-constructed graft. Even when the membrane is used as a surface allograft, the outer layer shows a marked resistance to vascularization from the graft bed (Avery & Hunt, 1967). This would tend to reduce the level of sensitization of the mother to any antigenic stimulus. On the other hand, this would not protect the membranes in a presensitized female, since it is known that vascularization is not required for the efferent arm of the immunological reflex (Merwin & Hill, 1954). Following the degeneration of the trophoblast giant cells and Reichert's membrane at approximately Day 14 of pregnancy, the yolk sac becomes exposed to the uterine tissues. By this time, however, the uterine epithelium has undergone regeneration and provides an unbroken front to the yolk sac. It is possible that this prevents entry into the uterine lumen of maternal immunologically competent lymphocytes to recognize any fetal membrane antigen, or of already sensitized lymphocytes to initiate an attack on the membrane. We are at present investigating this possibility by transplanting preparations of entire conceptuses with exposed yolk sacs to ectopic sites in allogeneic and presensitized recipients. The question of the susceptibility of this membrane to humoral antibody is also of interest, particularly in view of its involvement in the transmission of immunoglobulins from mother to young (Brambell, 1970). In allogeneic mouse pregnancy, there is known to be a significant alloantibody response to fetal (paternal) antigens (Kaliss, 1973), yet this has no observed effect on the apparently antigenic yolk sac. Heterologous antisera to kidney, placenta and yolk sac preparations, however, can have deleterious effects following injection into pregnant rats. Such antisera localize in the yolk sac, interfere with its function as an agent for embryonic nutrition, and secondarily produce severe fetal malformations (see Brent, 1971). Experiments in vitro involving the injection of sheep anti-rat yolk sac γ-globulin into different fetal membrane compartments of explanted rat embryos, or of addition to the culture medium, showed that only in the latter case, when it had contact with the endoderm layer of the yolk sac, was a teratogenic effect obtained (New & Brent, 1972). The endoderm thus appears to be the only fetal membrane component which is sensitive to the heterologous antibody. This is perhaps to be expected in view of its ability to absorb various macromolecules and initiate their transport to the embryo (Padykula, Deren & Wilson, 1966), but the effect may not necessarily have any immunological specificity. There appear to be no reports on the effect of alloantibody on the yolk sac, and our in-vitro preparations may provide a valuable method of approach.

The implications of investigations on the rodent yolk sac for understanding of fetal membrane function in man are not yet clear. In human development, the yolk sac is a significant structure only in the early stages, and is considerably
reduced by the time the placenta is established. The membrane most comparable in its anatomical relationships to the rodent yolk sac is the human chorion. This is essentially composed of a layer of trophoblast from two to ten cells thick, and is in intimate apposition to maternal decidua. Since implantation in man is interstitial, the embryo is not in the uterine lumen and there is no luminal epithelium between the chorion and the decidua. Whether the chorion is antigenic, and if so, how it escapes maternal immune aggression, is completely unknown. We have established that the outermost fetal membrane of the mouse is antigenic and susceptible to immune cell lysis in vitro. It cannot, therefore, act as a barrier to reactions of transplantation immunity between mother and fetus by those properties of resistance which we have recently shown to be exhibited by the early mouse trophoblast (Jenkinson & Billington, 1974). It remains to be seen whether, by virtue of its embryonic origin and cellular form, the human chorion is more akin to mouse trophoblast than yolk sac in its immunobiological nature.

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E. J. Jenkinson and W. D. Billington