DETECTION OF ALLOANTIGENS DURING PREIMPLANTATION DEVELOPMENT AND EARLY TROPHOBLAST DIFFERENTIATION IN THE MOUSE BY IMMUNOPEROXIDASE LABELING*

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The expression of antigenic determinants on the mouse embryo has been the subject of a number of investigations in recent years. Identification of these cell surface products of specific genetic loci is important for an understanding of the complex immunological interrelationships now known to exist between the maternal and fetal organisms (1, 2), for their use as markers in the analysis of tissue interactions in embryogenesis, and as indicators of gene activity during differentiation.

Earlier studies employing both transplantation and in vitro assays have demonstrated the presence of alloantigenic determinants from the two-cell stage (3–6). However, the mouse strain combinations used in these investigations differed both at the major (*H-2*) and minor (non-*H-2*) histocompatibility loci, and recent attempts have been directed towards a more precise characterization of these alloantigens. Immunofluorescence (7, 8), mixed antiglobulin, mixed agglutination (9), cytotoxicity (10), and transplantation (11) tests have all so far failed to detect H-2 alloantigens on the preimplantation embryo. These determinants have been reported to arise only in the postimplantation period, on putative inner cell mass cells present in blastocyst outgrowths developing in vitro (12), and on the embryonic, but not the trophoblastic, component of the 7½-day conceptus (13). In contrast, non-H-2 antigens have been detected on all cleavage stages and on blastocysts by a variety of techniques, including cytotoxicity (10), indirect immunofluorescence (7, 8), and ectopic transplantation (11, 14).

Despite these studies there are many issues still to be resolved. Further information is required on the precise nature and surface distribution of the alloantigens, and it is not yet clear whether the failure to detect H-2 reflects a true absence of these determinants or a lack of sensitivity of the techniques employed. In view of the apparent lack of antigenicity of the postimplantation ectoplacental cone trophoblast (15), a particularly crucial point to establish is the extent to which the outer layer of the preimplantation blastocyst, the trophectoderm, displays antigenic properties and how these change during its subsequent differentiation at the time of implantation in the allogeneic environment of the uterus.

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This communication reports the use of a sensitive immunoperoxidase technique, which allows examination of the cell membrane at the ultrastructural level, in an analysis of H-2 and non-H-2 antigen expression on cleavage stage embryos and during early trophoblast differentiation in the mouse.

Materials and Methods

Mice. The following inbred strains of mice were used: A(*H-2*^{a}), C57BL(*H-2*^{b}), C3H/He-mg (*H-2*^{k}), C57Br/cd(*H-2*^{k}), B10.Br(*H-2*^{k}), and C57BL/10ScSn (*H-2*^{b}). In differing combinations these animals provided embryonic material and antisera with major (H-2), minor (non-H-2), or combined (H-2 plus non-H-2) histocompatibility antigen differences.

Antisera

The procedure for production of antisera differed slightly in each of the three mouse combinations employed:

COMBINED H-2 AND NON-H-2. Adult male C57BL mice received two consecutive full-thickness skin grafts followed by four weekly intraperitoneal injections of 5×10^7 spleen cells from A strain donors. Animals were bled 6 days after the last injection, and the antisera were tested by hemagglutination.

NON-H-2. Adult C57Br mice were immunized against C3H tissues by one tail skin graft and six weekly intraperitoneal injections of 2×10^7 spleen and lymph node cells. Antisera were collected 7 days later and assayed for cytotoxicity against C3H lymphocytes.

H-2. C57BL/10ScSn recipients were immunized against the congenic strain B10.Br by five intraperitoneal injections of 2×10^7 spleen and lymph node cells at 10-day intervals followed by a single booster injection of 7×10^7 cells. Antisera were collected 7 days later and assayed for cytotoxicity against B10.Br lymph node cells.

Embryonic Material

Spontaneously ovulating female mice were caged with males overnight and examined for the presence of a vaginal plug the following morning. The day of plug detection was designated day 0 of pregnancy. Several stages of embryonic development from both pre- and postimplantation pregnancy were examined:

PREIMPLANTATION EMBRYOS. These were flushed from the reproductive tract in phosphate-buffered saline (PBS)[1] with 2% bovine serum albumin (BSA). Eight-cell stages were recovered from the oviducts on day 2 postcoitum (pc); morulae from the uterus on day 3 pc.; blastocysts from the uterus on day 3½ pc. The zona pellucida was routinely removed from the embryos by partial digestion with 0.5% pronase in PBS followed by mechanical disruption by repeated withdrawal into a glass micropipette. This latter procedure was carried out in pronase-free PBS to minimize exposure to the enzyme. In some cases zona removal was effected entirely by mechanical disruption.

PERI-IMPLANTATION EMBRYOS. To assess the antigenic status of the embryo in the critical period approaching and immediately after attachment to the maternal tissues two experimental procedures were adopted. Groups of pregnant females were bilaterally ovariectomised at day 2½ pc and given subcutaneous injections of 1-mg progesterone (Organon) in 0.1 ml arachis oil on alternate days for periods up to 10 days. This procedure prevents the blastocysts from implanting and maintains them in a state of experimentally controlled delay until activated to implant by subcutaneous adminstration of 0.1 μg 17-β-oestradiol (Organon Laboratories, Scotland) in 0.1 ml PBS (16). Removal of the blastocysts 18 h after oestrogen treatment allowed examination of the embryos at a time closely approaching implantation.

Since recovery of the embryo in the immediate postimplantation period is at present impractical and to examine implantation-associated changes in the trophoblast in the absence of complicating maternal factors, use was made of the ability of blastocysts to 'implant' and produce outgrowths in vitro. This is considered to be analogous to the initial stages of implantation in utero

[1] *Abbreviations used in this paper:* DAB, diaminobenzidine; FCS, fetal calf serum; HRP, horseradish peroxidase; ICM, inner cell mass; PBS, phosphate-buffered saline; pc, postcoitum; RaMIg, rabbit antimouse immunoglobulin antiserum.

(17). Zona-free blastocysts were transferred to RPMI culture medium plus 10% fetal calf serum (FCS) in Cooke microtitre plates (Cooke Laboratory, Alexandria, Va.) and maintained under 5% CO_2 in air for 3–4 days. During this period the blastocysts attached to the bottom of the culture vessel and produced outgrowths of trophoblastic cells together with some overlying cells derived from the inner cell mass.

POSTIMPLANTATION TISSUES. Entire conceptuses were dissected from the decidual shells of the 7½-day pregnant mouse uterus, placed in PBS and separated into ectoplacental cone trophoblast and embryonic sac (18). Care was taken to remove maternal decidual cells and Reichert's membrane surrounding the embryonic sac.

Peroxidase Labeling. This was performed in two ways. Initial studies were carried out using a method involving direct coupling of horseradish peroxidase (HRP) (Type VI, Sigma Chemical Co., St. Louis, Mo.) to the immunoglobulin fraction of C57BL anti-A antiserum with glutaraldehyde as the cross-linking agent according to the method of Avrameas (19). A strain embryos were maintained in RPMI plus 10% FCS at room temperature in the presence of 0.02 M sodium azide for 15 min and then cooled on ice for 10 min before the addition of ice-cold peroxidase-labeled antiserum to a dilution of 1:10 and futher incubation for 1 h at 0°C. The azide was employed to inhibit nonspecific uptake of label by pinocytosis (20). The embryos were washed extensively in PBS, fixed for 1 h in 2.5% glutaraldehyde in PBS, rewashed, and then incubated with a freshly saturated solution of 3,3′-diaminobenzidine (DAB, 0.3 mg ml^{-1}) and 0.01% H_2O_2 in 0.5 M Tris-HCl (pH 7.6) for 10–15 min at room temperature (21). After final washing the embryos were processed for electron microscopy as described below. C57BL embryos of equivalent stages were carried through in parallel as controls.

The remainder of the experiments were carried out using an indirect peroxidase-labeling technique. Rabbit antimouse immunoglobulin antiserum (RaMIg) was raised by immunizing rabbits by intramuscular injections of nonimmune mouse serum immunoglobulin emulsified in Freund's complete adjuvant. The immunoglobulin fraction was isolated from the RaMIg serum by ammonium sulphate precipitation and coupled with HRP as above. The precise conditions for the test varied with the alloantiserum used. With the C57BL anti-A and the C57Br anti-C3H antisera, the embryos were treated as above and incubated in ice-cold antiserum diluted 1 in 10 for 1 h, whereas the dilution of the C57BL/10ScSn anti-B10.Br antiserum was found to be optimal at 1 in 5. Embryos were then washed extensively, incubated with RaMIg-peroxidase conjugate for 1 h at 0°C, washed, and fixed for 1 h in 2.5% glutaraldehyde in PBS. After further washing the reaction for peroxidase activity was performed as described above for the direct labeling procedure.

The following controls were carried out in parallel as indicators for nonspecific reactivity: (*a*) embryos syngeneic with the strain in which the antiserum had been raised, (*b*) incubation of embryos in nonimmune serum, and (*c*) incubation with RaMIg-peroxidase conjugate alone.

Electron Microscopy. After treatment, preimplantation mouse embryos were deposited into a drop of molten 2% agar in PBS on a slide at 38–40°C. The agar was allowed to gel, and a small block containing the embryos was cut out for subsequent processing. In this form the embryos were postfixed in ice-cold 1% osmium tetroxide for 1 h, dehydrated, and embedded in Epon 812.

Except for the substitution of a 1:1 alcohol:Epon solution for propylene oxide, blastocyst outgrowths in microtitre wells were similarly postfixed and dehydrated and then embedded by addition of Epon directly into the well. After hardening of the Epon the plastic well was trimmed away to allow sectioning of the block. Postimplantation tissues were processed using conventional methods.

In most cases sections were not counterstained with lead tartrate or uranyl acetate since this was found to interfere with the high contrast between the 3,3′-DAB reaction product and the tissue background. Sections were examined in a Philips EM300 electron microscope (Philips Electronic Instruments, Mount Vernon, N. Y.) at an accelerating voltage of 80kV.

Results

Preimplantation Embryos. Visualization of surface antigenic determinants by binding of the electron-dense peroxidase conjugate was similar on all the preimplantation embryos where non-H-2 antisera and combined H-2 and non-H-2 antisera were employed (Table I). The pattern of labeling on the eight-cell,

TABLE I

*Detection of Alloantigens on Mouse Embryos by Immunoperoxidase Labeling**

Alloantisera	Antigenic difference	Preimplantation			Peri-implantation			Postimplantation (7½ days)	
		Eight-cell	Morula	Blastocyst	Activated blastocyst	Blastocyst outgrowth in vitro: ICM	Blastocyst outgrowth in vitro: Trophoblast	Embryonic sac	Ectoplacental cone trophoblast
C57BL anti-A									
Direct labeling	H-2 plus non-H-2	++	++	++	NT	NT	NT	++	–
Indirect labeling		++	++	++	++/–°	++	–	NT	NT
C57BR/cd anti-C3H (indirect labeling)	H-2 plus non-H-2	++	++	++	++/–°	++	–	NT	NT
C57BL/10ScSn anti-B10.BR. (indirect labeling)	H-2 only	–	NT	+	NT	++	–	++	–

++, heavily labeled; +, sparsely labeled; –, unlabeled; NT, not tested; °, labeling on activated blastocysts varied from heavily to unlabeled cells.

* Assessment based upon 5–15 embryos for each treatment.

morula, and 3½-day blastocyst was uniform and continuous over the cell surface (see Fig. 1). All controls were completely unlabeled. With the H-2 antisera, of the two stages of embryo examined, the eight-cell and blastocyst, only the latter showed labeling, but to a uniformly lesser intensity than with the other antisera (Fig. 2). This would appear to reflect a quantitative difference in the levels of H-2 antigen expressed at this time, a finding of particular interest in view of the inability of all previously reported techniques to detect these determinants (see Discussion).

The occurrence of peroxidase-containing pinocytotic vesicles immediately beneath the cell-surface, which was commonly observed when the labeling procedures were initially performed at room temperature, was prevented by incubation on ice in the presence of sodium azide. The only nonspecific labeling observed was on the cell surfaces lining the blastocoelic cavity, reflecting the ability of the blastocyst to accumulate macromolecules and the ineffectiveness of the washing procedures at this site.

Peri-implantation Embryos

OESTROGEN-ACTIVATED DAY 10 BLASTOCYSTS. Blastocysts recovered from the uterus after a 10-day period of experimentally-induced delay showed a significant and consistent reduction in the degree of peroxidase labeling with both the non-H-2 and the combined H-2 and non-H-2 alloantisera. Normal 3½-day blastocysts were all heavily labeled, and all controls were negative.

The distribution of the label on the blastocyst cell surface varied from a complete absence on some trophectoderm cells (Fig. 3) to a discontinuous but distinct labeling on other cells. The position of the labeled cells bore no apparent relation to the polarity of the activated blastocyst. It is clear that antigen loss occurs as the time for blastocyst implantation approaches.

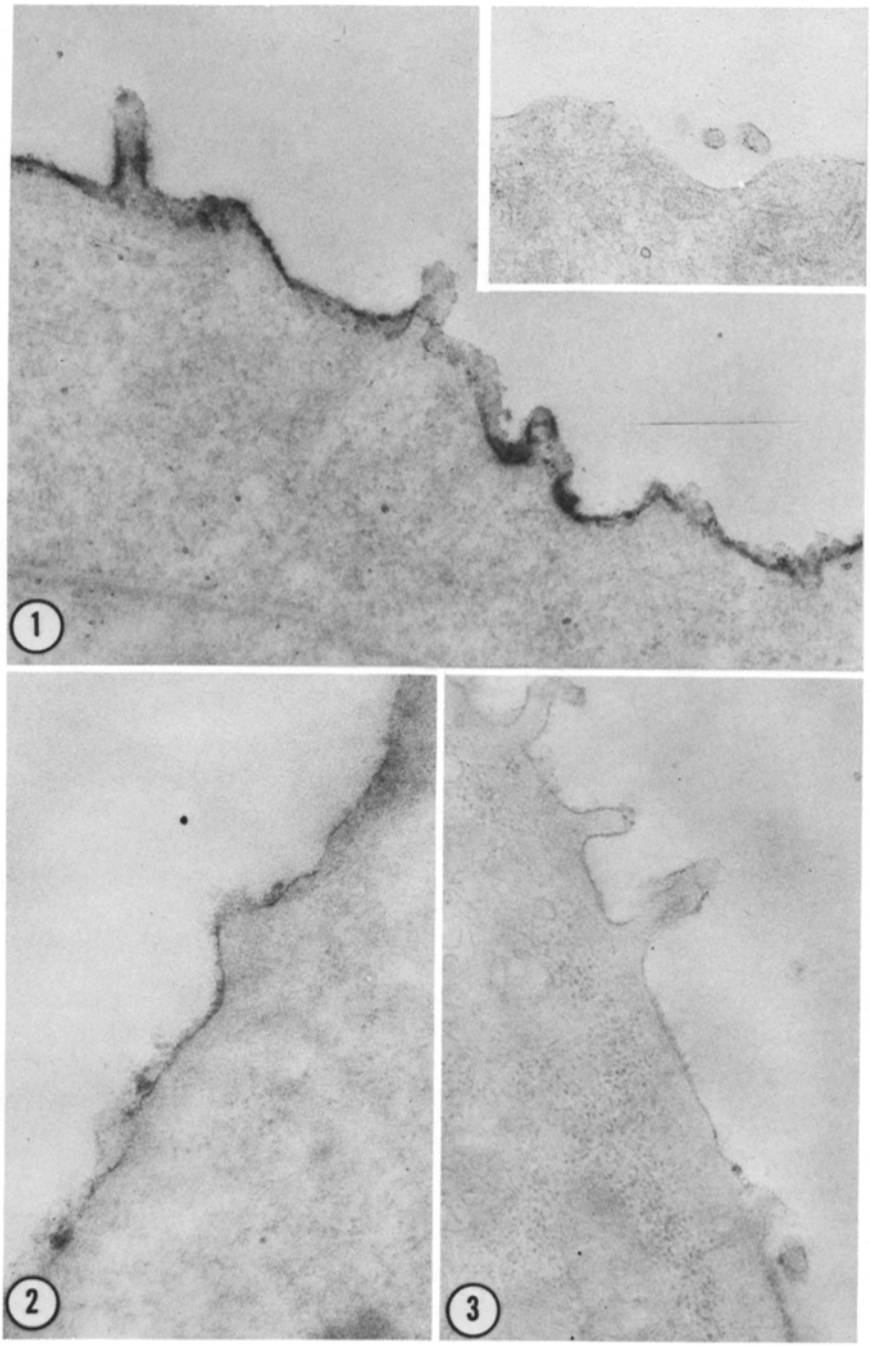

FIG. 1. Electron micrograph of the cell surface of a 3½-day A strain mouse blastocyst after immunoperoxidase treatment with antiserum directed against combined H-2 and non-H-2 antigens. The trophectoderm is covered by a continuous and heavy deposit of peroxidase conjugate. × 40,000. (Inset) Unlabeled surface of C57BL control blastocyst after similar treatment. × 40,000.

FIG. 2. B10. Br blastocyst after immunoperoxidase treatment with antiserum directed against H-2 antigen only. The labeling is discontinuous and less dense by comparison with that seen in Fig. 1. × 40,000.

FIG. 3. A strain blastocyst 18 h after experimentally-induced activation. The labeling has disappeared completely, indicating antigen loss at the time of implantation. × 40,000. (c.f. Fig. 1).

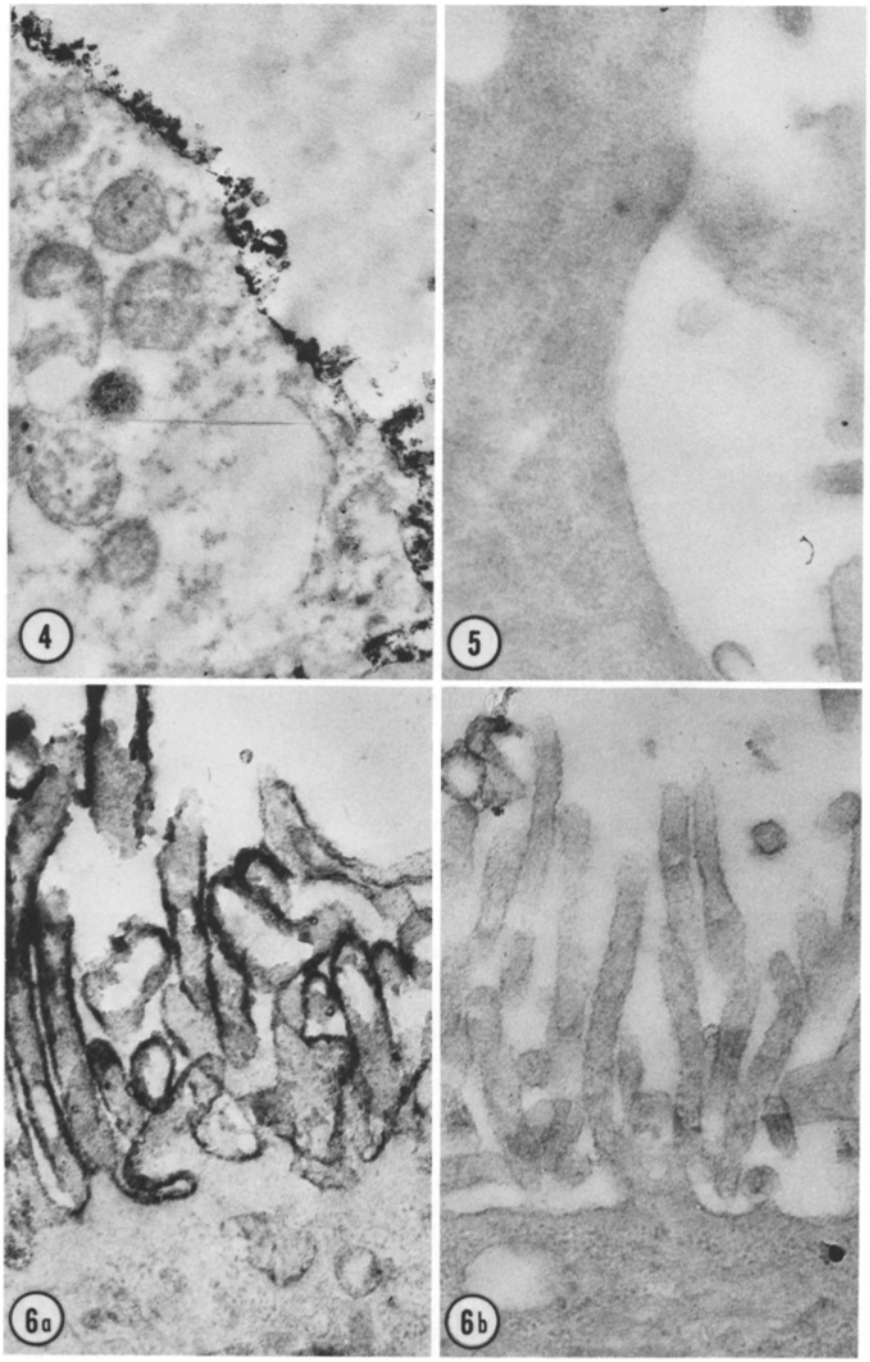

FIG. 4. In vitro outgrowth of C3H blastocyst after treatment with antiserum directed against non-H-2 antigens. The micrograph shows the surface of a cell derived from the inner cell mass of the blastocyst, with extensive labeling. × 14,000.

FIG. 5. Trophoblastic component of the same outgrowth as in Fig. 4, showing the complete absence of label on this cell type. × 40,000.

FIG. 6. (a) The highly microvillous cell surface of a 7½-day A strain embryonic sac after treatment with antiserum directed against combined H-2 and non-H-2 antigens. The labeling on this tissue is extremely dense, and occurs also in the cytoplasmic vesicles. (b) Unlabeled surface of control C57BL embryonic sac. Both × 40,000.

BLASTOCYST OUTGROWTHS IN VITRO. Blastocysts cultured in vitro for 3 days formed an outgrowth of trophoblastic cells derived from the trophectoderm and an overlying group of smaller cells presumably of inner cell mass origin. These two cell types displayed a striking difference in their surface labeling properties after incubation with the various antisera. The embryonic cells were densely labeled with continuous deposits of peroxidase (Fig. 4), while the trophoblastic cells were always entirely unlabeled (Fig. 5). The use of the outgrowth model thus provided a means of examining the early stages of differentiation of the trophoblast and provided further evidence for the loss of demonstrable antigenic determinants at this critical stage in the implantation process.

POSTIMPLANTATION CONCEPTUS. Examination of the two main components of the 7½-day mouse conceptus, the ectoplacental cone trophoblast and the embryonic sac, confirmed the differential antigenic status indicated by the results of the blastocyst outgrowth study (Table I). The embryonic sac, derived from the inner cell mass, possesses an outer endodermal layer that was densely labeled after antiserum treatment (Fig. 6). It was also noted that those endodermal cells situated on the lateral walls of the sac showed evidence of peroxidase label within large cytoplasmic vesicles, whereas those at the posterior tip of the sac did not. The cells at the tip were lacking in any obvious cytoplasmic vacuole activity and possessed few cell surface microvilli. In all control embryos the cell surface was unlabeled.

In contrast to the embryonic cells, the trophoblastic cells on the outer regions of the ectoplacental cone showed no evidence of peroxidase labeling in either experimental or control groups. With the present technique no conclusions can be drawn about the antigenic status of any cells within the central portion of the ectoplacental cone. Although the lack of staining of any of the trophoblastic cells of the blastocyst outgrowths might argue to the contrary, it has been suggested that there may be two distinct populations of cells in the ectoplacental cone with differing antigenic properties (22). Immunoperoxidase tests on cone outgrowths in vitro or on sectioned material could resolve this point.

Discussion

The results of these investigations confirm earlier reports (7, 8, 10) that the cleavage stage mouse embryo expresses minor histocompatibility antigens (non-H-2), but not those determined by the major locus (*H-2*). In marked contrast to previous studies, however, H-2 as well as non-H-2 antigens have been found on the trophectoderm of the ensuing stage of development, the preimplantation blastocyst, although the pattern of labeled antiserum binding observed suggests that the expression of these H-2 antigens is relatively weak at this stage. The discrepancy between the present findings and those of previous workers using immunofluorescence probably reflects the greater resolution of the electron microscopical immunoperoxidase technique, and it might be expected that other indicators of appropriate sensitivity, such as radiolabeled antisera, would also reveal the presence of these antigens on the blastocyst. Similarly, low antigen levels, as well as the transient nature of their appearance on the trophectoderm (see below), may explain the failure to detect H-2 antigens in experiments involving ectopic transplantation of blastocysts to preimmunized recipients (11).

The present detection of H-2 antigens on the trophectoderm of the preimplantation blastocyst represents the earliest stage at which these products of the *H-2* locus have been demonstrated on the fertilized mouse embryo. It has been reported that H-2 antigens can be identified on the unfertilized mouse ovum (23), but it would appear that as cleavage proceeds these are either diluted out or lost, and that further H-2 antigens do not appear in detectable levels until the blastocyst stage is reached, suggesting a differential activity on the part of genes controlling H-2 and non-H-2 antigen expression before this time. At present, however, it is unclear whether the apparent lack of H-2 antigens on the cleavage stages is due to the complete repression of their synthesis or whether they are produced but in some way fail to be expressed, possibly as a result of being synthesized in an incomplete form. Recent reports have suggested that certain antigens present on at least some forms of teratoma, and also detectable on the preimplantation embryo, have a reciprocal relationship with H-2 antigen in their expression on the cell surface and disappear by the time H-2 becomes detectable on the postimplantation embryo, implying that they may be precursors of the complete *H-2* molecule (24). So far the validity of this hypothesis and the level at which the switchover from precursor to definitive H-2 antigen synthesis is effected remains to be established.

As far as the postimplantation stages are concerned, transplantation and in vitro cytotoxicity tests using embryos explanted at 7½ days have shown that a differential antigenicity exists between the trophoblast of the ectoplacental cone, which appears to have no detectable antigens, and the inner cell mass (ICM)-derived egg cylinder, which expresses H-2 and non-H-2 antigens (15, 25, 26). In all these studies the actual developmental age of the tissues at the time of antigen detection is difficult to determine because of the possibility of continued differentiation between the time of explantation and the time of assessment. The results of the present investigation, however, clearly indicate that the differential antigenicity of the trophoblastic and embryonic components of the conceptus, with respect to both H-2 and non-H-2, exists at the 7½-day stage.

The precise time of appearance of H-2 antigens on the embryonic derivatives of the inner cell mass has yet to be established. Experiments involving ectopic transplantation of postimplantation embryos have suggested that these antigens are not present on the 6-day egg cylinder, but arise 1–1½ days later (13). This is substantially in agreement with the immunofluorescence studies of Heyner (12) showing that H-2 is first detectable on putative ICM cells of blastocysts that have been allowed to 'implant' and continue their development in vitro to a stage considered to be equivalent to day 6½ in utero. It remains to be seen whether the low levels of H-2 detectable on the trophectoderm by immunoperoxidase are also present on the ICM at the blastocyst stage. Examination of ICM's isolated by microdissection to ensure adequate exposure to the labeled antisera should resolve this point. At whatever time H-2 antigens first appear, however, it is apparent that their expression only increases to more readily detectable levels with the onset of the rapid processes of differentiation and embryogenesis which are not initiated until implantation is established.

In view of the apparent lack of antigens (both H-2 and non-H-2) on the postimplantation ectoplacental cone trophoblast, as compared to the preimplan-

tation trophectoderm, it would seem that during the course of its differentiation antigen expression on the trophoblast is in some way abolished. As indicated by the comparison of preactivated blastocysts with those activated to implant in vivo or allowed to 'implant' in vitro, this antigen loss occurs concurrently with the initiation of implantation, a finding consistent with a recent report on changes in specific antibody-binding to the blastocyst at implantation as assessed by mixed hemadsorption and isotope antiglobulin techniques (27). The precise mechanism by which this antigen loss takes place remains uncertain, although the in vitro experiments suggest that the process is intrinsic to the blastocyst and not necessarily dependent upon maternal factors. At the time of implantation the cells of the trophectoderm undergo considerable modification, including increasing polyploidy (28), the acquisition of marked phagocytic properties (29), and changes in ultrastructure indicative of increased metabolic activity (30), in membrane ultrastructure (31) and in membrane glycoproteins[2]. It seems reasonable to consider that the loss of surface antigens may be related to these extensive alterations in the physiology and membrane properties of the trophoblast.

In biological terms the disappearance of trophoblast surface antigens at implantation could be highly advantageous with respect to the survival of the fetal allograft. During its early development the embryo is enclosed within the zona pellucida which, although it may be permeable to antibody (32), should provide an effective barrier to immunologically competent maternal cells. At implantation, however, this acellular coat is shed and the blastocyst attaches to the uterine epithelium which soon degenerates, allowing close contact of the trophoblast with the uterine stroma and maternal blood elements. Under these conditions the ability of the embryo to present an outer surface deficient in antigens, at a time when antigen expression on the inner cell mass is probably increasing, should reduce the possibilities of recognition or immunological attack by the maternal immune system. In this way it may be possible to explain the ability of the fetal allograft to establish itself during the crucial period when other protective mechanisms, e.g. serum blocking factors, are unlikely to be operative and when, being relatively small, the embryo would be easily overwhelmed by a maternal immune response.

It should also be noted that the presence of histocompatibility antigens on the preimplantation embryo, particularly those of the major *H-2* complex, may have implications for the immunological manipulation of early pregnancy, especially in view of the evidence that paternal antigens are expressed during these stages of development (8). In as much as the appearance of antigens on those components of the embryo directly exposed to the maternal environment is relatively transient, however, the time when such interference is possible may be strictly limited. In this respect further information on the immunological status of the uterine environment in both the pre- and peri-implantation periods is required, especially in those species, such as man, where implantation is of the intimate interstitial type.

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[2] Jenkinson, E. J., and R. F. Searle. 1976. Cell surface changes on the mouse blastocyst at implantation. Submitted for publication.

Summary

An immunoperoxidase-labeling technique allowing visualization of antibody binding to the cell surface at the electron microscopical level has been employed in an analysis of H-2 and non-H-2 alloantigen expression on the early mouse embryo. The presence of non-H-2 antigenic determinants has been confirmed on eight-cell, morula, and blastocyst stages of development. Contrary to previous reports, however, low levels of H-2 antigen have also been detected on the blastocyst. This is the earliest stage at which H-2 has been shown to be expressed on the fertilized mouse egg and may reflect the greater resolution of the immunoperoxidase technique.

Using two different models to study the critical peri-implantation stages, those of experimentally induced blastocyst activation and blastocyst outgrowth in vitro, it has been demonstrated that antigen loss occurs on the trophectoderm at the time of implantation, and that this is not necessarily dependent upon maternal influence. It is suggested that the loss may be an important factor in the prevention of maternal immune rejection during the establishment of the fetal allograft.

The two major components of the early postimplantation conceptus display a striking differential in antigenic status. The embryonic sac shows a high degree of peroxidase labeling, while the ectoplacental cone trophoblast is unlabeled. These findings add support to the concept of antigenic neutrality of the early trophoblast and its role in the maintenance of a normal fetomaternal immunological equilibrium.

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