Placentation in the mouse involves both a chorioallantoic disk in contact with maternal blood and a visceral yolk sac projecting into the uterine lumen. Neither of these placental tissues shows any sign of immunological rejection during pregnancy, even in females previously sensitized to paternal strain alloantigens. The protective mechanism for the yolk sac is probably not simply its position in the uterine lumen, because allografts in privileged positions—such as the anterior chamber of the eye and the hamster cheek pouch—are destroyed when the host is sensitized (1). Previous studies have shown that the mouse yolk sac is rejected when grafted to an allogeneic host (2, 3). Also, its endodermal epithelial cells, which are in contact with maternal tissue in vivo, are susceptible to the cytotoxic action of alloreactive lymphocytes when the epithelial cells are dissociated from the yolk sac and maintained in culture (4). In attempting to reconcile the presence of target alloantigens on the surface epithelial cells with the failure of sensitized females to react against the yolk sac in vivo, Jenkinson and Billington (4) noted that the entire surface of the dissociated epithelial cells in vitro was exposed to alloreactive cells, whereas in the intact epithelial layer in vivo only the apical membrane is exposed to the mother. They suggested the possibility that there might be a polarized distribution of alloantigens on yolk sac epithelial cells, such that they were expressed in the laterobasal membrane but not in the apical membrane in contact with maternal tissue.

This suggestion is now supported by recent immunoferritin-labeling studies of the distribution of major histocompatibility complex (MHC)\textsuperscript{1} antigens on adult-lining epithelial cells. On 12 such cell types, dissociated from tissues after prefixation to preserve the native antigen distribution it was observed that mouse H-2-complex antigens were expressed variably in the laterobasal membranes but were never detected in the apical membranes (5–8). The failure to detect H-2 antigens in the apical membranes was not a result of masking by other membrane components, and, when epithelial cells were dissociated without prefixation, there was a migration of H-2 molecules from the laterobasal membranes into the apical membranes (6). On prefixed, intestinal-absorptive cells, the H-2 antigens were absent from the zonula occludens membrane but were present in high concentration in the adjacent zonula adherens membrane, which indicates that the zonula occludens portion of the epithelial junctional complex is the barrier that normally prevents the mixing of apical and laterobasal membrane components (6). Secretory component (9) and

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\textsuperscript{1}Abbreviations used in this paper: BSA, bovine serum albumin; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; PLP, periodate-lysine-paraformaldehyde.
specific I-region antigens (10) have also been detected in the laterobasal membranes of intestinal cells, but not in the apical membranes. Thus, evidence is accumulating that lining epithelial cells have two different kinds of surface membrane, one at the apical end and another at the laterobasal end, kept separate by the zonula occludens (11-13). From the evolutionary point of view, the advent of higher organisms required the development of the tight junction to keep the internal environment separate from the contents of the body cavities. Once having such a barrier, it would seem inevitable that the membranes facing the two different environments would become uniquely specialized. In the case of MHC antigens, their presence in the laterobasal membranes in contact with the internal environment, which is self, and their absence from the apical membranes adjacent to the outside world, which is not-self, obviously complements the theory that these molecules function as self-markers for the immune system (14).

The self-side distribution of MHC antigens on lining epithelial cells is relevant to the maternal-fetal relationship. Mammalian embryonic development occurs within a closed sac of one or more fetal membranes that are covered by an epithelial layer oriented with its self-side toward the embryo and its not-self-side toward the mother. A confinement of fetal MHC antigens to the self-side of placental epithelium would apparently deprive the mother of target sites for an alloimmune reaction. Both Jenkinson and Billington (4) and Johnson (15) have previously recognized that such a polarized distribution of fetal MHC antigens on placental epithelium would have important implications for the maternal-fetal relationship. To determine whether there may be a polarized distribution of H-2-complex antigens on mouse visceral yolk sac placental epithelial cells we have studied the apical and laterobasal membranes of these cells by immunoferritin labeling, complement-mediated cytotoxicity, and cell-mediated cytotoxicity.

### Materials and Methods

**Mouse Strains and Antiserum.** Mice of the congeneric strains C57BL/10Sn (H-2b) and B10.BR/SgSn (H-2k) were obtained originally from The Jackson Laboratory, Bar Harbor, Maine, and are now maintained at The University of Adelaide (Adelaide, South Australia). C3H/HeJ mice were obtained from the Institute of Medical and Veterinary Sciences, Adelaide, Australia. Alloantiserum against the complete H-2k complex was produced in B10.BR/SgSn × C3H/HeJ F1 hybrid females by immunizing with C57BL/10Sn spleen cells, as described previously (6). This antiserum had a titer of ~1/60 by hemolysis assay with rabbit complement (16). Antiserum against the Ia antigens of the H-2k haplotype, prepared by immunizing mice of strain A.TH with lymphoid cells from strain A.TL, was generously donated by Dr. Ian McKenzie, Department of Medicine, Melbourne University (Melbourne, Australia). This antiserum was adsorbed with spleen cells from mice of strain B10.D2 (H-2d), from Dr. McKenzie's colony in Melbourne, and used on B10.BR/SgSn cells as positive with B10.D2 cells as the congeneric control, as described previously (10). This antiserum had a cytotoxic titer of ~1/1,000. For the cell-mediated cytotoxic assay, mice of strain C57BL/10 and the congeneric strain B10.G (H-2d) were obtained from the John Curtin School of Medical Research, Australian National University, Canberra, Australia.

**Yolk Sac Preparations.** Pregnant female mice were killed on the 16th and 20th d of pregnancy. Their uteri were removed, placed in cold Hanks' balanced salt solution, and cut open along the antimesometrial side to expose the embryos enclosed in the amnion and yolk sac. The chorioallantoic placental disks were peeled away from the decidua, the fetal membranes were incised, and the embryos were removed. The amnion and yolk sac membranes were then trimmed away from the placental disks and collected in cold Hanks' solution. The washed fetal membranes were minced briefly with scissors and suspended in Hanks' solution that contained...
5 μg/ml DNAase (Sigma type II; Sigma Chemical Co., St. Louis, Mo.). The tissue was incubated for 10 min at 37°C, then placed on ice, and allowed to sediment at 1 g for 4 min. Two further brief washes at 1 g in cold Hanks' solution removed the remaining erythrocytes and tissue debris. The washed fetal membranes were then either prefixed in periodate-lysine-paraformaldehyde (PLP) (17) for 3 h at 4°C or transferred to phosphate-buffered saline (PBS) that contained 1% bovine serum albumin (BSA). Prefixation of mouse tissue in PLP has no detectable effect on the specific labeling of H-2 complex (18) or specific Ia (10) antigens. The prefixed tissue was washed once in PBS before being transferred to PBS-BSA. Under microscopic observation, individual pieces of the villous portion of the fixed or unfixed yolk sac preparations were picked up by pipet and transferred to siliconized test tubes or culture wells, as appropriate.

Immunoferritin Labeling. Pieces of fixed or unfixed yolk sac on days 16 and 20 of pregnancy, from C57BL/10Sn and B10.BR/SgSn mice for the complete H-2 complex, and from B10.BR/SgSn and B10.D2 mice for Ia antigens, were processed in siliconized test tubes for immunoferritin labeling as described previously (6, 10). The apical membrane of yolk sac endodermal epithelial cells was exposed to the labeling reagents in these preparations. Some preparations of prefixed day-20 C57BL/10Sn yolk sac membranes were mixed with fixed, resident peritoneal cavity cells of the same strain to provide an internal, positive labeling control for the H-2 complex. Other preparations of fixed day-20 C57BL/10Sn yolk sac were exposed to sequential digestion by neuraminidase and trypsin under conditions that lead to a substantial removal of the visible fuzzy filaments from jejunal-absorptive cells (6). This was done to remove possible masking substances from the apical membrane of yolk sac epithelial cells and thus to expose possibly hidden H-2-complex molecules. Additionally, prefixed C57BL/10Sn and B10.BR/SgSn yolk sacs from the 20th d of pregnancy were dissociated, either with EDTA and trypsin or with trypsin alone. The dissociation procedure has no effect on the specific labeling of H-2-complex antigens (18). The resulting cell suspensions contained mainly endodermal epithelial cells and vascular endothelial cells, which were exposed to the labeling reagents on all their surfaces. The labeled cells and yolk sac pieces were further processed for electron microscopy as described previously (6). In addition, a few pieces of yolk sac were prepared for scanning electron microscopy by standard methods.

Complement-mediated Cytotoxicity. Yolk sac pieces from single day-20 C57BL/10Sn donors for each experiment were incubated in 200 μl of normal mouse serum plus 200 μl of complement, or in the same volumes of whole H-2-complex alloantiserum and complement. The mouse sera were diluted 10-fold with medium 199, whereas adsorbed rabbit complement was prepared as described by Boyse et al. (19) and used undiluted. After incubation for 60 min at 37°C and washing in PBS, cell viability was assessed by trypan blue staining as described by Philips (20). Also, day-20 C57BL/10Sn yolk sac preparations were dissociated with 0.02% EDTA and 0.25% trypsin in PBS. The cells released during several 10-min incubations at 37°C were dispersed into cold Hanks' solution that contained 20% heat-inactivated fetal calf serum, then pooled and washed in Hanks' solution. Each cell suspension was divided into two 100-μl aliquots for incubation in 100 μl of undiluted complement and 100 μl of 1/10 dilution of either H-2-complex antiserum or normal mouse serum for 60 min at 37°C. After washing in PBS, cell viability was determined by trypan blue staining. Positive controls for the complement-mediated cytotoxic test were provided by C57BL/10Sn peritoneal cavity cells incubated in antiserum and complement or in normal serum and complement, followed by washing and trypan blue staining, and by C57BL/10Sn erythrocytes either incubated in the antiserum and complement mixture or suspended in it and then immediately centrifuged without incubation. The sizes of the erythrocyte pellets and the optical density of the supernates at 540 nm were assessed.

Cell-mediated Cytotoxicity. B10.G anti-C57BL/10 alloreactive cells were generated in a primary in vitro response as described previously (21). Single small yolk sac pieces from one day-20 C57BL/10Sn donor for each experiment were incubated in RPMI-1640 culture medium that contained 10% heat-inactivated fetal calf serum and 20 mM Hepes buffer in 0.2 ml culture wells at 37°C under 5% CO₂ and air. One-half of the wells received 2 × 10⁶ alloreactive cells that were immediately centrifuged down to form a pellet surrounding the piece of yolk sac. After 1, 2, and 3 h of incubation, the medium was changed in all wells, and the cells were suspended and resedimented around the yolk sacs to encourage diverse contacts between killer
cells and target cells. After 5 h of incubation, all yolk sac pieces were washed in PBS and stained with trypan blue. The pieces incubated with alloreactive cells were compared with those incubated without added cells. Alloreactive cells from the same batches used on the yolk sacs were also incubated for 5 h with C57BL/10 macrophage targets as a positive control, cytotoxicity being monitored in this case by chromium release, as described previously (21).

Results

Apical Membrane of Endodermal Epithelial Cells. The pieces of mouse visceral yolk sac that we selected for this study had well-developed villi covered by an intact layer of endodermal epithelium in which the maternally oriented, apical cell membranes were exposed to the medium for labeling and for the cytotoxic tests (Figs. 1 and 2). Immunoferritin labeling of these apical membranes with or without pre-fixation failed to detect any antigens of the whole H-2 complex or the specific I-region, nor were any antigenic sites unmasked by neuraminidase and trypsin treatment before labeling (Fig. 3). The labeling density of H-2-complex antigens on the peritoneal macrophages added to some yolk sac preparations as an internal, positive labeling control was ~75 ferritin molecules/10-cm length of membrane on prints at a magnification of 64,000 (Fig. 4), whereas on the apical membranes of yolk sac epithelial cells there was ~1

Fig. 1. This scanning electron micrograph shows the elaborate villous structure of the maternal side of a piece of mouse visceral yolk sac at the 20th d of pregnancy. The villi are uniform in height and have similar widths, but their length and orientation are variable. The endodermal epithelial cells covering the villi are variable in size, and their apical membranes bulge outward. × 320.
Fig. 2. This light micrograph of the yolk sac at the 20th d of pregnancy shows the simple columnar layer of endodermal epithelium covering the villi. The apical surface of these epithelial cells is normally in contact with uterine tissue and luminal fluid, and, in our experiments, it was exposed to the cells or reagents in the incubation medium. The endodermal epithelium constitutes a large part of the mass of the tissue, which enabled us to use trypan blue staining to obtain an estimate of the proportion of the endodermal cells killed in the cytotoxic tests. The villi contain thin cores of vascularized (V) mesoderm, and there is a squamous layer of mesothelial cells on the fetal side of the tissue. The structure of the mouse yolk sac is quite similar to that of the rat yolk sac described previously (22, 23). × 500.

ferritin molecule/10-cm length of membrane in both the antigenic and the congenic control mouse strains. The incubation of yolk sac pieces in anti-H-2-complex alloantiserum and rabbit complement had no effect on the number of epithelial cells stained by trypan blue. Fewer than 10% of the cells were stained after incubation in alloantiserum and complement or normal mouse serum and complement, or in pieces of yolk sac obtained directly from the minces and not incubated at all. Yolk sac preparations whose cells were killed by incubation in 1% acetic acid for 20 min or by storage in Hanks' solution at 4°C for 8 h showed extensive epithelial cell death by trypan blue staining. The alloantiserum and complement caused complete and specific lysis of peritoneal cell targets under the same conditions used on the yolk sac pieces, and caused substantial hemolysis of erythrocytes with conversion of ~0.5 the incubated cells into ghosts. The incubation of single small pieces of yolk sac in 0.2-ml round-bottom culture wells with 2 × 10⁶ alloreactive lymphoid cells for 5 h also had no effect on the number of epithelial cells stained by trypan blue. Fewer than 10% of the cells were stained in pieces incubated with or without the addition of alloreactive lymphoid cells. The alloreactive cells gave virtually complete killing of target macrophages in the same time period at a ratio of one killer: one target. The number of yolk sac target cells was not known, but the small pieces of yolk sac were embedded
MOUSE YOLK SAC PLACENTA LACKS H-2 COMPLEX ALLOANTIGENS

Fig. 3. The apical membrane of endodermal epithelial cells exhibits numerous, irregular microvilli (MV). The sample illustrated here from the 20th d of pregnancy was prefixed in PLP and exposed to reagents for immunoferritin labeling of the whole H-2 complex. There is no detectable ferritin binding. The inconspicuous, fuzzy coat on the apical membrane could be substantially removed from PLP-fixed tissue by neuraminidase and trypsin treatment, but this did not unmask any H-2-complex antigens. Ferritin did not penetrate through the junctional complexes (JC) to reach the intercellular space between epithelial cells. × 64,000.

Fig. 4. Resident peritoneal cavity cells were mixed with pieces of yolk sac during immunoferritin labeling of H-2-complex antigens to provide a positive, internal-labeling control. The macrophage illustrated here is from one such preparation, and shows the typical pattern of dense ferritin (F) binding to the surface membrane that is characteristic of the H-2-complex labeling of these cells. × 64,000.
in a larger mass of alloreactive cells during incubation. In addition, the preparations were suspended and resedimented after 1, 2, and 3 h of incubation to promote cell contact.

Laterobasal Membrane of Endodermal Epithelial Cells. Cell suspensions obtained by dissociation of PLP-fixed yolk sac preparations, either with EDTA and trypsin or with trypsin alone, contained mainly endodermal epithelial cells and a few vascular endothelial cells. The entire surface membrane of the dissociated cells was exposed to labeling reagents. H-2-complex antigens were not detected by immunoferritin labeling on either the apical or the laterobasal membranes of the endodermal epithelial cells in any of the preparations (Fig. 5), but they were detected on the vascular endothelial cells. The antigen density on the unlabeled endodermal epithelial cells could not have been more than ~1% of what we find on peritoneal macrophages, and on the vascular endothelial cells the labeling density was less than we have observed previously on adult endothelial cells from thyroid glands (8) and islets of Langerhans (7). The cell suspensions obtained for the complement-mediated cytotoxic test by dissociation of unfixed yolk sac tissue also contained mainly endodermal epithelial cells. These were recognizable in the light microscope without staining because they are larger than any other yolk sac cell type. There was no attempt to remove dead cells from these preparations, and, consequently, after control incubation in normal mouse serum and complement, ~75% of the cells stained with trypan blue. The viable cells were mainly endodermal epithelium. When the original cell suspension was divided equally and one-half was incubated in the alloantiserum and complement described above for apical membranes and the other one-half was incubated in normal mouse serum and complement, there was no detectable difference between the two preparations after trypan blue staining, either in the proportion of cells remaining viable, which was ~25% in both samples, or in the proportion of the viable cells that were endodermal epithelium. Thus, although the background of dead cells was high, there was no indication of any specific killing of freshly dissociated endodermal epithelial cells by complement-mediated cytotoxicity.

Fig. 5. A portion of the lateral membrane of an endodermal epithelial cell from the 20th d of pregnancy is shown. The cells were dissociated from prefixed yolk sacs with EDTA and trypsin and then exposed to labeling reagents for the complete H-2 complex. There is no detectable ferritin binding. In the same preparations there were vascular endothelial cells that showed a low density of ferritin binding. × 64,000.
Discussion

Mouse H-2-complex antigens are variably expressed in the laterobasal membranes of epithelial-lining cells but appear to be absent from the apical membranes (5-8). This polarized expression of MHC molecules in the laterobasal self-side membranes adjacent to the internal environment but not in the apical membranes adjacent to the outside world is consistent with the function of these molecules as self-markers for the immune system. The present study was an attempt to extend these observations to a placental epithelium, where the apical membranes are oriented toward the mother. An absence of fetal self-marker molecules from the apical membranes of placental epithelium would deprive the mother of target sites for an alloimmune reaction at the maternal-fetal interface. Our results support this suggestion. We were unable to detect H-2-complex antigens in the apical membrane of mouse yolk sac placental epithelial cells by three techniques. The apical membrane was unaffected by alloreactive lymphocytes that killed all target macrophages, or by a complement-mediated cytotoxic test that killed all peritoneal cavity cells and caused significant hemolysis of erythrocytes. Immunoferritin labeling indicated that the apical membrane could contain no more than ~1% of the antigen that is present on peritoneal macrophages. Because the apical membrane of endodermal epithelial cells is the only fetal cell membrane exposed to the pregnant female in the yolk sac placenta, the deficiency of fetal alloantigens in that membrane is probably an important factor in the failure of specifically sensitized females to react against the yolk sac in vivo.

We also could not detect H-2-complex antigens in the laterobasal membrane of endodermal epithelial cells dissociated from yolk sacs. Immunoferritin labeling indicated that this membrane could contain no more than ~1% of the H-2 antigen that is present on macrophages, and the complement-mediated cytotoxic test had no effect on the dissociated cells. It has been reported that EDTA can extract H-2 antigens from embryonic tissues (24), but when trypsin alone was used for cell dissociation we were still unable to detect H-2 antigens in the laterobasal membrane by immunoferritin labeling. In contrast, Jenkinson and Billington (4) found that dissociated yolk sac endoderm cells in culture for 1 d were destroyed by alloreactive lymphocytes during the next 2 d in culture. The authors suggested that the target antigens might have been present in the original laterobasal membranes of the cells. This suggests that there may be a small amount of H-2 antigen in the laterobasal membrane that can be detected by cell-mediated cytotoxicity but not by complement-mediated cytotoxicity or immunoferritin labeling. However, it is also possible that the dissociated cells, after several days in culture and the loss of their normally polarized structure, could have undergone a differentiation that included the new expression of H-2 antigens. Thus, it remains uncertain whether H-2-complex antigens occur in the laterobasal membrane of the yolk sac epithelium. If they are present, their density is quite low.

The deficiency of MHC molecules in the laterobasal membrane of yolk sac epithelium may be related to a generally lower expression of MHC antigens in fetal mouse tissues than in the corresponding adult tissues (25). This possibility is supported by our observation that the endothelial cells of vitelline vessels in the yolk sac expressed a significantly lower density of H-2-complex antigens than adult endothelial cells from islets of Langerhans (7) and thyroid glands (8). The deficiency may also be related to the observation that H-2 antigens cannot be detected by immunoferritin
labeling in the laterobasal membranes of several adult epithelial cell types, such as parietal cells, tracheal-ciliated cells, and vas deferens epithelium (6). The significance of the absence or low density of H-2 antigens on such adult cells is not known. The adult epithelial cell type most closely related to the yolk sac endoderm is the absorptive cell of the small intestine, and this cell shows a dense expression of MHC molecules in its laterobasal membrane (6, 10).

When junctional complexes are present in a placental epithelium and only the apical cell membranes are exposed to the mother, as in the yolk sac epithelium, the composition of the laterobasal membrane is probably not an important factor in the failure of sensitized females to react against that epithelium. The zonula occludens portion of the junctional complex separates and maintains the unique compositions of the two membrane regions, and an absence of fetal self-markers from the apical membrane would deprive the mother of target alloantigens regardless of the composition of the laterobasal membrane. If maternal lymphocytes penetrated through the junctional complexes to gain access to the laterobasal membranes, they would simultaneously gain access to all of the fetus. This appears not to occur to any significant extent because offspring are not normally tolerant of unshared maternal alloantigens and the offspring of mothers sensitized against paternal alloantigens do not normally suffer from graft-vs.-host symptoms (26). However, where the organization of the placental epithelium is less regular, it may be important that the entire surface of the epithelial cells lacks MHC molecules. Examples would include rodent chorionic giant cells and human syncytiotrophoblast, which are often scattered and fail to form a continuous layer, and cells in the human cytotrophoblastic shell, which form a multicellular layer. Faulk and Temple (27) have shown convincingly by fluorescent antibody labeling of frozen sections that the human syncytial and cytotrophoblastic epithelial layers lacked detectable HLA antigens on all parts of the cell surface, whereas the vascular endothelium in the villus cores was obviously labeled. And Ferguson and Palm (28) could not detect MHC antigens on rat chorionic giant cells. However, this point remains controversial because it has also been reported that mouse H-2-complex antigens were present on cells that were thought to be chorionic epithelium (29).

Summary

The only fetal cell membrane exposed to the mother in the mouse yolk sac placenta is the apical membrane of the endodermal epithelial cells. In yolk sac preparations in vitro, this apical membrane was exposed to reagents or cells in the incubation medium. By using several techniques we were not able to detect fetal major histocompatibility complex (MHC) antigens in this membrane. Immuno ferritin labeling with and without prefixation and after neuraminidase and trypsin digestion indicated that the apical membrane could contain no more than ~1% of the H-2 complex antigens that were present on peritoneal macrophages. Incubation of yolk sac preparations in anti-H-2 complex antiserum and complement had no cytotoxic effect on the endodermal epithelium, nor did incubation in an excess of alloreactive lymphocytes. Dissociated preparations of prefixed yolk sac contained endodermal epithelial cells and vascular endothelial cells whose entire surface membranes were exposed to the medium. H-2-complex antigens were not detected by immuno ferritin labeling in either the apical or the laterobasal membrane of the yolk sac endoderm, but they were present in low
density on the vascular endothelium. Also, incubation of unfixed, dissociated cells in anti-H-2-complex serum and complement had no detectable cytotoxic effect on endodermal epithelial cells. These observations indicate that H-2 antigens are sparse or absent in both the apical and laterobasal membranes of endodermal epithelial cells. The deficiency of MHC antigens in the apical membrane may account for the failure of sensitized females to reject the yolk sac, whereas the composition of the laterobasal membrane is probably less important to maternal-fetal relations. The present observations are consistent with labeling studies of adult-lining epithelial cells, which indicate that self-marker MHC molecules are absent from the apical membranes oriented toward the outside world and variably expressed in the laterobasal self-side membranes. It is suggested that the corresponding exclusion of fetal self-marker molecules from the apical membranes of some kinds of placental epithelia would deprive the mother of target sites for an alloimmune reaction at the maternal-fetal interface.

Received for publication 9 June 1980.

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