LOCALIZATION OF PATERNAL H-2K ANTIGENS ON MURINE TROPHOBLAST CELLS IN VIVO*

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The fetoplacental unit resulting from an allogeneic mating is a natural allograft that is not rejected by the otherwise immunocompetent mother (1, 2). Of the various hypotheses proposed to explain this phenomenon, the possible absence of major histocompatibility antigens from the surface of the fetally derived trophoblast cells which lie at the fetomaternal interface has been the focus of extensive investigation (3–11).

We have recently demonstrated (10) the presence of H-2K and D antigens of both maternal and paternal haplotypes on the surface of murine trophoblast cells in suspensions of collagenase-dispersed placentae from 9 to 18 d of gestation, using monospecific antisera and a highly sensitive radioautographic technique. The antigen density on 12- and 14-d trophoblasts was comparable to that on adult F_1 thymocytes and a further 50% increase was observed at term. However, the question still remained as to whether these antigens are expressed *in situ*, and if so, whether they are detectable on the face of trophoblast cells that line the maternal sinusoids in vivo, and thus accessible to the maternal circulation. Studies by Kirby and Cowell (11) and Parr et al. (12) have demonstrated that H-2 antigens may be sequestered on one aspect of certain cells, e.g., the epithelial cells of the small intestine and the endoderm cells of the yolk sac.

This study was designed to examine the presence of H-2K antigens on the sinusoidal surface of murine trophoblast cells in vivo, without destroying the architecture of the intact placenta. The use of a radioautographic technique, after an in vivo perfusion with radioiodinated antibodies via individual placental branches of the uterine artery, ensured high levels of sensitivity. High levels of specificity were provided by the use of monoclonal antibodies raised against the H-2K antigens of the paternal haplotype. The visualization and quantitation of radioautographic silver grains on morphologically identified trophoblast cells allowed good resolution. The results revealed significant levels of H-2K antigens of the paternal haplotype on the sinusoidal face of mouse trophoblast cells in the labyrinthine region of placentae derived from allogeneic matings.

Materials and Methods

Mice. C57BL/6J females were mated with CBA/J or C57BL/6J males (6–12 wk of age; The Jackson Laboratory, Bar Harbor, ME) and provided allogeneic and syngeneic placentae,

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respectively, at day 15 of gestation. The day of appearance of a vaginal plug was counted as day 0 of pregnancy.

Sera. Monoclonal anti-H-2K^k (Becton, Dickinson & Co., Sunnyvale, CA) was used. A protein A column-purified IgG_{2a} fraction of tissue culture supernatant was obtained. This antibody has been previously characterized (13).

Radioiodination. Radioiodination of monoclonal anti-H-2K^k antibodies was carried out by a modification of the chloramine T oxidation method of Greenwood et al. (14). Briefly, 25 μg protein A affinity column-purified anti-H-2K^k antibody was incubated with 1.1 mCi of $Na^{125}I$. The reaction was started with 3.5 mM chloramine T, and after 10 min was stopped with 10.8 mM potassium metabisulfite. Unbound ^{125}I was separated from the radioiodinated protein on a column of Sephadex G-25 (Pharmacia Inc., Uppsala, Sweden). This procedure yielded a specific activity of 50 μCi/μg of antibody.

In Vivo Perfusion. Placentae were perfused by the cannulation of individual placental branches of the uterine artery using a Venoset 78 infusion set (Abbott Diagnostics, North Chicago, IL) and a Sage infusion pump (model 355, Sage Instruments Div., Orion Research Inc., Cambridge, MA). The initial perfusion was done with phosphate-buffered saline, pH 7.4, to clear the blood from the maternal blood vessels and sinusoidal spaces at a flow rate of 0.2 ml/min until individual placentae were blanched. Fig. 1 shows the appearance of three fetoplacental units in the uterus following this procedure. Note that the placenta is blanched and a blanched band circles the fetus, indicating the vascular supply of the uterus. After this, each placenta was in turn perfused with radioiodinated monoclonal anti-H-2K^k antibody at a concentration of 2 μg/ml for 3 min at the same flow rate. This flow rate proved to be optimal for the maintenance of placental architecture, as evaluated histologically. After this, placentae were again perfused with buffer for 5 min to clear any unbound antibody. Finally, they were fixed either by a perfusion of the whole organ or by an immersion of 1-mm^3 pieces in 2.5% phosphate-buffered glutaraldehyde.

Histology. Fixed placentae were dehydrated in graded ethanols and propylene oxide and embedded in epon. Semi-thin (0.5-μm thick) sections were cut and stained with iron-hematoxylin and processed for radioautography (15). Sections mounted on slides were coated with NTB-2 nuclear emulsion (Eastman Kodak Co., Rochester, NY) and exposed for 21, 30, 45, 60, and 75 d. A 45-d exposure was found to be optimal for good labeling of cells with low levels of background grains.

Evaluation of Radioautographs. Sections were scored under the light microscope using an

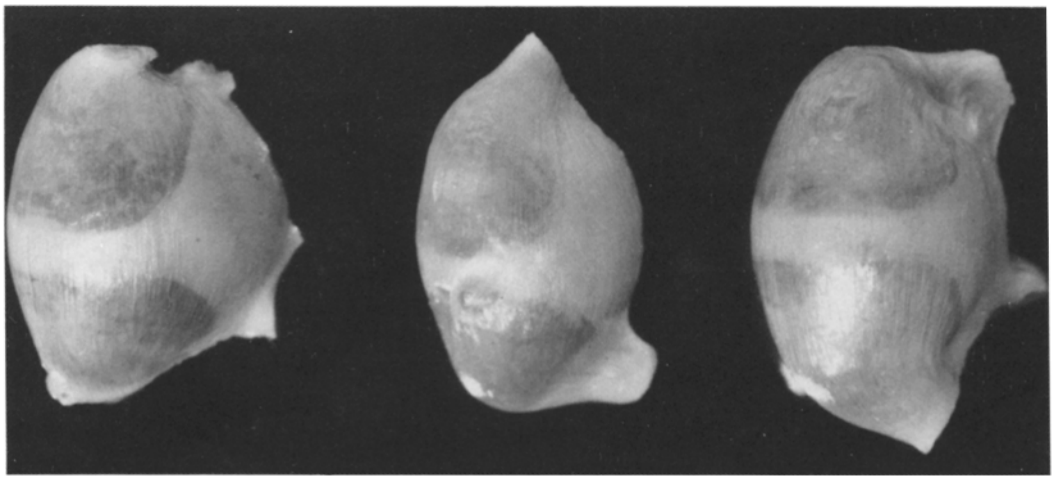

FIG. 1. Fetoplacental units with surrounding uterus after local perfusion via individual placental branches of the uterine artery. The blanched area includes the placenta and an equitorial band around the fetus, indicating passage of perfusate. The absence of blanching of the fetus indicates that fetal circulation is unaffected.

eyepiece micrometer grid. Grains were counted per unit area of 64 μm^2. The results are expressed as the absolute number of grain counts per unit area without any subtraction of background grains. The grain count distribution on the lumen of maternal sinusoids served as an indicator of background. At least 1,000 unit areas of 64-μm^2 were scored over trophoblast cells. For other cell types, sinusoidal lumina, and Reichert's membranes, 1,000 areas were scored where possible. Otherwise the largest number of available unit areas in 8–10 sections was scored. Data were pooled from 25–30 placentae from syngeneic or allogeneic pregnancy.

Results

Morphological Evaluation. Radioautographic preparations of histological sections were evaluated to examine the silver grain count distribution not only over the various cell types in the placenta, but also over various areas on trophoblast cells from different regions of the placenta shown in Fig. 2. The hemochorial placenta may be divided into three main zones: (*a*) The spongiotrophoblast zone (Fig. 3 C), which lies closest to the maternally derived decidua. The trophoblast cells of this layer are large, often multinucleated, and contain abundant cytoplasm. There are fewer sinusoidal spaces in this layer than in the labyrinthine zone. (*b*) The labyrinthine zone (Fig. 4 A, B) where maternal blood vessels open into a large number of sinusoidal spaces that are lined by trophoblast cells. These cells are often multinucleated and have many attenuated processes lining the maternal sinusoids. It is in this layer that an extensive surface area of trophoblast cells is exposed to maternal blood. (*c*) The giant cell layer, which consists of a layer of very large and multinucleated trophoblast cells lying at the periphery of the placenta (Fig. 4 D). Part of this layer remains in contact with the Reichert's membrane. In the 15-d mouse placenta, this layer is relatively thin. Furthest from the maternal tissues is the yolk sac endoderm, which lies on the embryonic side

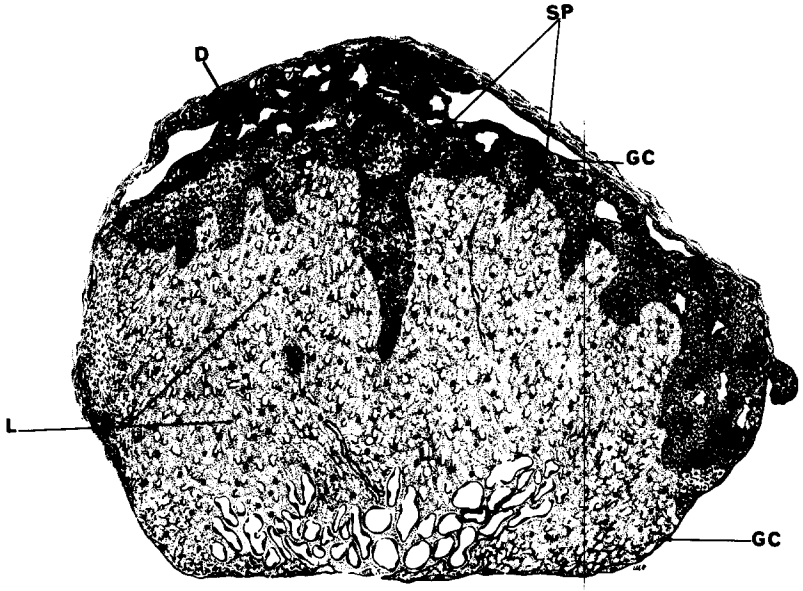

FIG. 2. A diagrammatic representation of a cross-section through the murine placenta. D, decidual tissue; SP, spongiotrophoblast cells. Note cords of spongiotrophoblast cells extending into the labyrinthine zone; L, labyrinthine zone. Note the large number of sinusoidal spaces; GC, trophoblast giant cells.

of the Reichert's membrane. It consists of rounded basophilic cells with dark nuclei (Fig. 3 D).

Distribution of Label in the Placenta. (Figs. 3, 4 and Table I). The mean grain density of the sinusoidal lumen in allogeneic placentae (0.59 ± 0.01) was assumed to represent the background. Although this may represent an overestimation of the actual background for all areas of the placenta, the choice of a maximum possible threshold was considered desirable to evaluate the presence of H-2 antigens. With this threshold, labyrinthine trophoblasts from allogeneic placentae, macrophages, the Reichert's membrane from both allogeneic and syngeneic placentae, and the endothelium of fetal capillaries of the allogeneic placenta labeled above background levels. Moreover, each of these cell types and the Reichert's membrane from allogeneic placentae showed significantly greater labeling than those from syngeneic placentae, at a difference significant at $P < 0.005$. However, spongiotrophoblasts from either allogeneic or syngeneic placentae do not show labeling above background. For example, the mean grain densities are 0.22 ± 0.003 and 0.16 ± 0.001, respectively, which were less than those in the corresponding labyrinthine sinusoidal lumen.

Macrophages from allogeneic placentae had a mean grain density of 5.41 ± 0.14 as compared with 0.93 ± 0.12 on those from syngeneic placentae. Some of these macrophages were seen within sinusoids (Fig. 3 D), and others were found in the stroma around fetal capillaries. The latter may represent the equivalents of Hoffbauer cells seen in the human placenta.

The endothelium of the fetal capillaries in allogeneic placentae had 3.47 ± 0.13 grains per unit area as compared with the complete absence of grains from those in syngeneic placentae. The Reichert's membrane from allogeneic placentae (Fig. 3 D) had a mean grain density of 2.02 ± 0.02 as compared with 1.29 ± 0.01 in syngeneic placentae, a difference that is significant at $P < 0.005$. However, it should be noted that the grain density on the Reichert's membrane from syngeneic placentae (Fig. 4 D) is substantially higher than our estimate of the background, indicating some nonspecific binding of the radiolabeled antibodies.

The decidual cells of the uterus, fetal leukocytes, and erythrocytes, the endodermal cells of the parietal yolk sac, and the trophoblast giant cells that lie immediately adjacent to the Reichert's membrane did not show labeling above background levels. Thus, the degree of significance of the differences of the grain densities on these cell types from allogeneic and syngeneic placentae, although shown in Table I, are of little biological relevance.

Localization of H-2 Antigens (Table II). To determine if specific binding of radiolabeled antibodies could be localized to the sinusoidal face of the labyrinthine trophoblasts, the cells were divided into two components: the sinusoidal face, defined as a 1-μm thick band along the membrane lining the sinusoids, and intracellular aspect, which repesented areas over the rest of the trophoblast cell. The results shown in Table II indicate that the mean grain density on the sinusoidal face of the trophoblast cells from allogeneic placentae was 8.5 ± 0.0025 as compared with either 1.3 ± 0.006 on the intercellular aspects of the same cells, or 1.7 ± 0.01 on the sinusoidal face of trophoblasts from syngeneic placentae. These results indicate that most of the radiolabeled antibody was bound specifically to H-2K^k molecules of the paternal haplotype that must be present on the sinusoidal face of trophoblast cells.

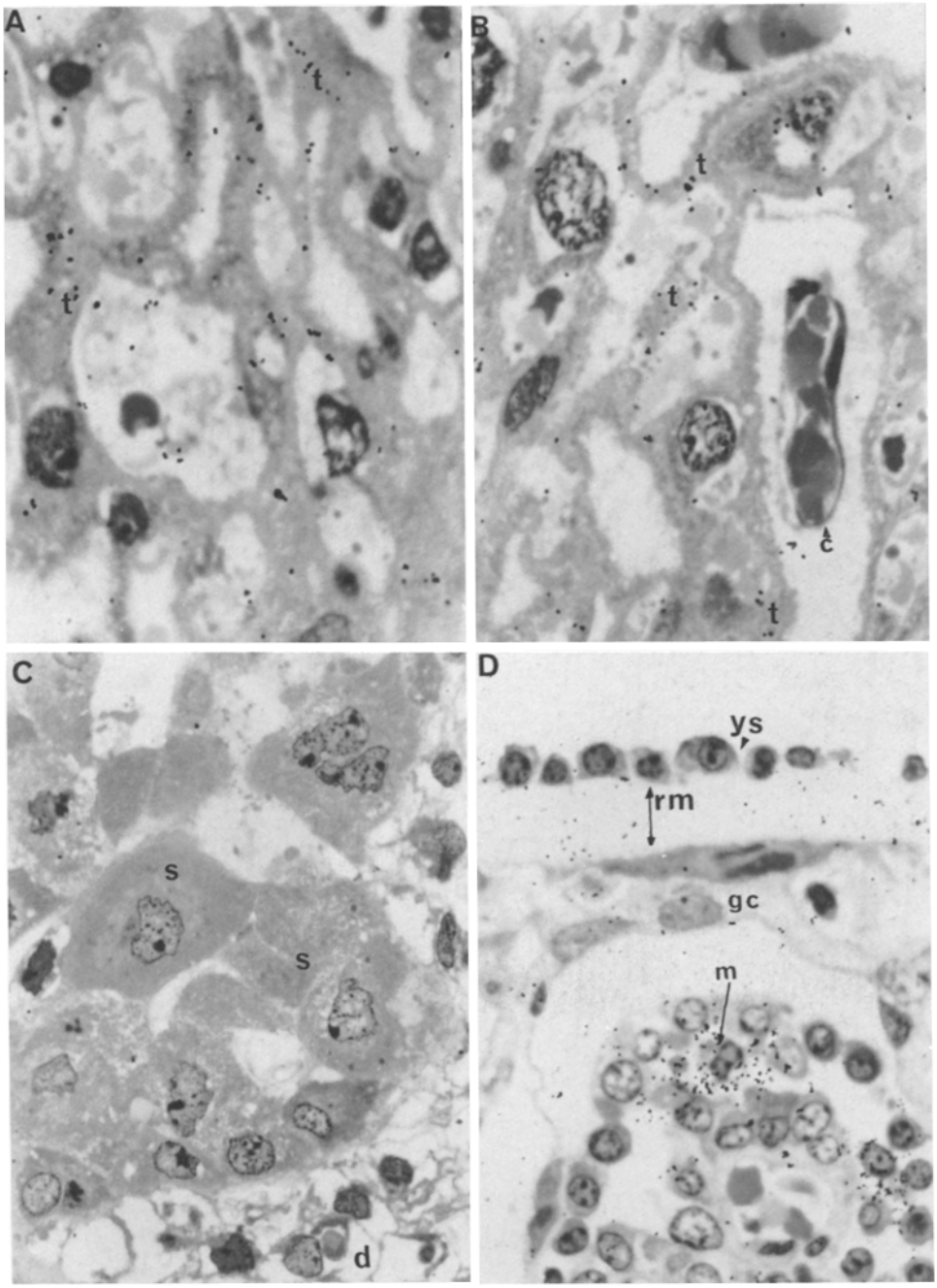

FIG. 3. Radioautographs of sections through (CBA/J × C57BL6/J)F_1 placentae after local perfusion of radiolabeled monoclonal anti-H-2K^k antibodies. (0.5-μm thick sections stained with iron-hematoxylin.) (A, B) Section through the labyrinthine zone (× 2,600). t, trophoblast cells. Note silver grains on trophoblast cells lining the sinusoids, indicative of paternal H-2K antigens; c, fetal capillary. (C) Section through spongiotrophoblast zone with adjoining maternally derived decidua (× 2,600). s, spongiotrophoblast cell; d, decidual tissue. Note the paucity of silver grains on both cell types. (D) Section through the lateral region of the placenta (× 2,200). ys, parietal yolk sac; rm, Reichert's membrane; gc, trophoblast giant cells; m, macrophage (within a sinusoid). Note heavy labeling of macrophage.

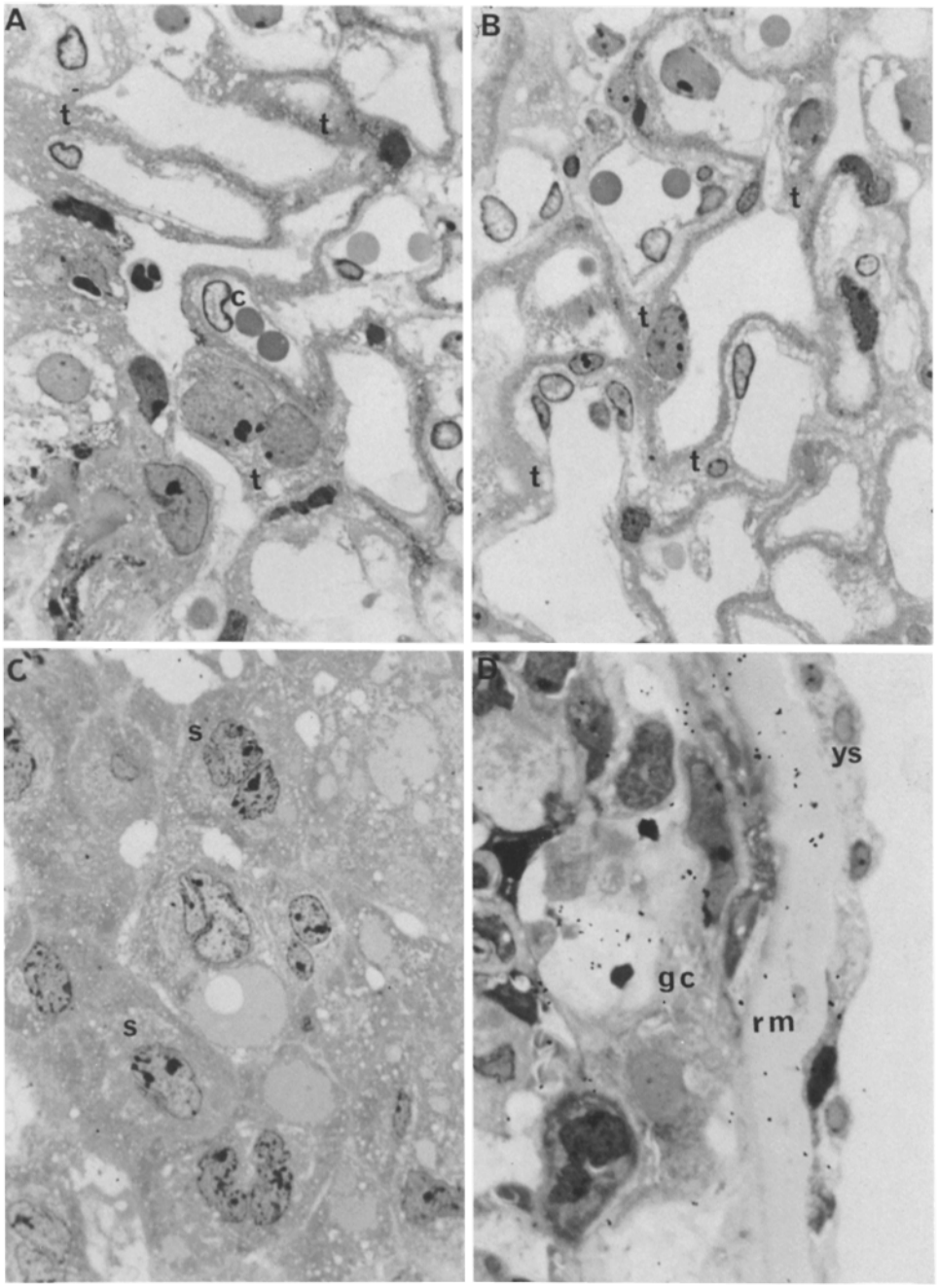

FIG. 4. Radioautographs of sections through syngeneic C57BL6/J placentae after local perfusion with ^{125}I-labeled monoclonal anti-H-2K^k. (0.5-μm thick sections, stained with iron-hematoxylin.) (A, B) labyrinthine zone (× 2,200). t, trophoblast cells; c, fetal capillary. Note the lack of labeling of the various cell types for H-2K^k antigens. (C) spongiotrophoblast zone (× 2,200). s, spongiotrophoblast cells. Note paucity of label. (D) lateral area of placenta (× 2,600). ys, parietal yolk sac; rm, Reichert's membrane; gc, trophoblast giant cells. Note nonspecific labeling of the Reichert's membrane.

TABLE I

Grain Density (per unit Area of 64 μm^2) of Different Areas of the Placenta after In Vivo Infusion of ^{125}I-Anti-H-2K^k

	Allogeneic pregnancy (Mean ± S.E.)	Syngeneic pregnancy (Mean ± S.E.)	Level of significance
Decidual cells	0.16 ± 0.002	0.36 ± 0.01	$P < 0.005$
Sinusoidal lumen	0.59 ± 0.01	0.22 ± 0.01	$P < 0.005$
Spongiotrophoblasts	0.22 ± 0.003	0.16 ± 0.001	$0.05 < P < 0.1$
Labyrinthine trophoblasts	2.05 ± 0.004	0.24 ± 0.001	$P < 0.005$
Trophoblast giant cells	0.58 ± 0.01	0.58 ± 0.01	$P \gg 0.45$
Macrophages	5.41 ± 0.14	0.93 ± 0.12	$0.005 < P < 0.01$
Reichert's membrane	2.02 ± 0.02	1.29 ± 0.01	$P < 0.005$
Yolk sac endoderm	0.3 ± 0.126	0.7 ± 0.04	$0.025 < P < 0.01$
Fetal endothelium	3.47 ± 0.13	0	$P < 0.005$
Fetal leukocytes and erythrocytes	0.65 ± 0.02	0.02 ± 0.003	$P < 0.01$

TABLE II

Grain Density over Different Areas of Labyrinthine Trophoblasts

	Allogeneic pregnancy	Syngeneic pregnancy	Level of significance
Sinusoidal face of trophoblast cells	8.5 ± 0.025	1.7 ± 0.01	$P < 0.005$
Intracellular aspect	1.3 ± 0.006	0.115 ± 0.002	$P < 0.005$

Discussion

In this study we have examined the presence of H-2K antigens of the paternal haplotype on the surface of trophoblast cells in vivo using radioiodinated monoclonal anti-H-2K^k antibodies with specificity for the paternal haplotype. A morphological evaluation of the results enabled us to determine the specificity and pattern of binding of the antibodies to trophoblasts and other cells of the placenta. Additionally, we were able to localize specific binding of the antibodies to the sinusoidal face of trophoblast cells of the labyrinthine layer of the mouse placenta. Although light microscopy does not permit resolution to the level of the plasma membrane, the specific binding observed is attributable primarily to the presence of H-2 antigens on the plasma membrane.

The antibodies were delivered via the maternal sinusoids without disrupting the placental architecture, and hence allowed for an assessment of the accessibility of the blood-borne components of the maternal immune system to the H-2 antigens on the surface of trophoblast cells. Thus, H-2 antigens on the labyrinthine trophoblasts are not sequestered away from the sinusoidal face.

A paucity of the binding of radiolabeled antibodies to the spongiotrophoblasts may have resulted from a lower accessibility of the antibodies to the cells in question, due to a smaller surface area being exposed to maternal sinusoids rather than a lack of H-2 antigens. H-2 antigens have been detected on spongiotrophoblasts in vitro by Jenkinson and Owen (16). The absence of positive and specific labeling of the trophoblast giant cells at the periphery of the placenta, despite the perisinusoidal location of some of these cells, indicates that they may lack detectable levels of H-2 antigens.

The endothelial cells lining the fetal capillaries in allogeneic placentae exhibited specific labeling for H-2 antigens of the paternal haplotype. The access of antibodies to these cells is probably a result of transport across trophoblast cells (17), followed by specific binding. These results also indicate that a short period of 15–25 min is sufficient for the passage of antibodies from the maternal sinusoids to the fetal capillaries. Moreover, the antigen-binding sites of these molecules must be intact and unoccupied to exhibit specific binding to the fetal endothelium.

Macrophages facing the sinusoidal lumen in both allogeneic and syngeneic placentae labeled above background levels, indicating some nonspecific binding, probably via Fc receptors. However, the extent of labeling of macrophages was sixfold in allogeneic placentae, indicating that most of these macrophages were probably of fetal origin. The observation that some of these macrophages are seen within the sinusoidal lumen would indicate that they have migratory properties.

Due to the uniqueness of the hemodynamics and the vasculature of the placenta, plus the relationship of the trophoblast cells to the sinusoidal spaces, it was impossible to use another organ as a positive control that would afford a similar passage of such large amounts of arterial contents through such extensive sinusoidal spaces lined by the target cells in question. However, positive labeling of the fetal endothelium may serve as an internal positive control.

The labeling of the Reichert's membrane above background levels in syngeneic placentae probably reflects nonspecific binding of immunoglobulins. Whether the higher labeling observed in allogeneic placentae may be attributed to the presence of $H\text{-}2K^k$ molecules or to the binding of antigen-antibody complexes remains to be determined. The latter represents a strong possibility, because immune complexes are known to bind to other basement membranes such as that of renal glomeruli.

After an intravenous administration of radiolabeled anti-H-2 antibodies with specificity for the paternal haplotype, Wegmann and Carlson (18) have reported a preferential accumulation in allogeneic placentae on the basis of whole placental radioactivity. Recently, they claim to have localized such binding to spongiotrophoblast cells (unpublished results) 1–6 h after a systemic injection, as opposed to our observations 20 min after a local perfusion.

Although preferential sequestration of H-2 antigens in some tissues has been documented (11, 12, 19), this is not seen in the labyrinthine trophoblasts. Thus the allogeneic fetoplacental unit survives despite the presence of H-2K antigen, a very strong immunogen, on the face of trophoblast cells that lies in direct contact with maternal blood in vivo, and also appears to escape immunological assault when implanted on ectopic sites in allogeneic hosts (20–22). We have recently shown (23) that trophoblast cells do not express detectable levels of class 2 MHC (Ia) antigens. The important role of class 2 antigens in maximizing a rejection response in vitro as well as in vivo has been previously documented (24, 25). Organ allografts, when depleted of passenger leukocytes (26) by either a prior in vitro culture or ALS treatment (27, 28), survive longer, presumably due to the depletion of Ia-bearing leukocytes.

However, the similarities between the survival of trophoblasts and Ia-depleted thyroid parenchymal allografts extends only to their survival in unsensitized hosts. Upon sensitization, thyroid parenchymal allografts are rejected (27), because functionally mature cytotoxic T cells can recognize class 1 antigens on the cells of the

graft and destroy them. Although a few investigators (29, 30) have reported the destruction of trophoblasts after sensitization in vivo or in vitro, many (20–22) have found the persistence of trophoblast cell grafts in allogeneic recipients. This suggests an unusual refractoriness of the trophoblast cell to lysis. This may be a result of (*a*) an ineffective presentation of class 1 antigens. It is possible that the class 1 antigens on trophoblast cells, although detectable by antibodies, may not present a certain epitope necessary for effective recognition by alloreactive T cells. Such an epitope may not necessarily be detectable by antibodies; or (*b*) a local inhibition of effector cells, possibly mediated by trophoblasts themselves, via mediators such as progesterone (31, 32) or other factors that have not yet been characterized.

Summary

We have previously shown the presence of H-2K and D antigens of both parental haplotypes on dispersed murine trophoblast cells. The question still remained whether such antigens are sequestered away from the sinusoidal face of these cells making them inert as allografts. The in vivo expression of H-2 antigens on these cells was therefore examined radioautographically after perfusion of ^{125}I-labeled monoclonal and anti-H-2K^k (anti-paternal) antibody directly into individual placental branches of the uterine artery suppling 15-d-old (C57BL/6J♀) × CBA/J♂) placentae. Syngeneic C57BL/6J placentae served as negative controls. A radioautographic examination of 0.5-μm-thick sections revealed specific labeling of labyrinthine trophoblasts lining the sinusoids of allogeneic placentae. Most of this labeling was localized to the sinusoidal face of the cells as opposed to a weak labeling of the intracellular aspect. Spongiotrophoblasts and trophoblast giant cells did not label, but specific labeling of fetal capillary endothelium and some macrophages was also noted.

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