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Induction of class I major histocompatibility complex antigen expression by West Nile virus on γ interferon-refractory early murine trophoblast cells

(implantation/trophoblast giant cells/flaviviruses)

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ABSTRACT **Primary murine trophoblast giant cells (TGC) do not express detectable major histocompatibility complex (MHC) antigens and are refractory to the MHC-increasing effects of α and β (virus-induced) interferons and γ (immune type) interferon during early implantation (postcoital days 3.5–6). West Nile virus infection of primary TGC monolayers from postcoital-day-3.5 preimplantation blastocysts induced paternal MHC antigen expression within 16 hr, as detected by immunogold labeling for electron microscopy. Induction is unlikely to have been mediated by secreted virus-induced interferons or other factors, as it occurred in the presence of high concentrations of anti-α/β interferon antibodies and was not induced by virus-inactivated supernatants from MHC-induced primary TGC cultures. Attempts to induce MHC antigen expression with poly(I·C) or recombinant tumor necrosis factor α in primary TGC cultures also failed. Thus, the apparent inhibition of MHC antigen expression in primary TGC during early implantation and their refractoriness to induction of *de novo* MHC antigen expression is not absolute. This may represent a maternal- and/or species-protective evolutionary device. As such, manipulation of this phenomenon may allow a conclusive assessment of the significance of inhibition of MHC antigen expression on trophoblast cells in the implanting semiallogeneic embryo.**

The semiallogeneic mammalian embryo survives gestation without immunological rejection, although placental cells expressing paternal major histocompatibility complex (MHC) antigens could theoretically be recognized and killed by maternal alloimmune cytotoxic T (T_c) cells. Thus, absence or low expression of MHC antigens on trophoblast cells during implantation has been suggested as a strategy for evading the maternal cellular immune response (1, 2).

The preimplantation murine blastocyst (3, 4) and trophoblast cells of the established placenta (5) express MHC antigens, but neither primary nor secondary trophoblast giant cells (TGC) express detectable MHC antigen. γ-Interferon (IFN-γ) induces paternal class I MHC antigen expression *de novo* on secondary but not primary TGC (6, 7) and increases class I MHC antigen expression on trophoblast cells from mature placentas (5). This suggests a programmed inhibition of MHC gene transcription during implantation (6–8).

Infection of several embryonic cell types with a range of flaviviruses, in particular West Nile virus (WNV), causes an increase in class I, and in some cases class II, MHC antigen expression. This increase depends on host-cell mRNA synthesis, is not blocked by anti-α/β interferon (anti-IFN-α/β) antibodies, and is functionally reflected by an increased susceptibility to lysis of infected cells by both WNV-immune and alloimmune T_c cells (9, 10).

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We report here that WNV induces the *de novo* expression of paternal class I MHC antigens in MHC-negative primary TGC during the IFN-γ-refractory period when implantation occurs. Thus, the apparent inhibition of MHC antigen expression in primary TGC is not absolute during this time. This may reflect a maternal- and/or species-protective evolutionary device but could be most useful to investigate the control of implantation.

MATERIALS AND METHODS

Animals. Specific pathogen-free C57BL/6J ($H\text{-}2^b$) and CBA/H ($H\text{-}2^k$) mice were bred at the John Curtin School of Medical Research. Virgin females 8–10 weeks old were placed with males overnight at a ratio of 2:1 and examined the following morning for vaginal sperm plugs. The day on which the plug was found was designated day 0 postcoitum (pc).

Cell Culture. Day-3.5-pc and -7.5-pc C57BL/6J and (C57BL/6J × CBA/H)F_1 blastocysts and ectoplacental cones (EPC) were isolated and placed in culture as described (6, 7).

Mouse embryo fibroblasts (MEF) were obtained from C57BL/6J or (C57BL/6J × CBA/H)F_1 fetuses as described (7, 11) and used at the third passage.

Virus Stocks. Vero cells were grown in Dulbecco's modified Eagle's medium (no. 430-1600, GIBCO), supplemented with 5% (vol/vol) fetal calf serum, 200 μg of streptomycin per ml, 200 units of penicillin G per ml, and 125 μg of neomycin sulfate per ml (DMEM) in Nunclon Delta 175-cm^2 tissue culture flasks (no. 56502) in humidified 95% air/5% CO_2 at 37°C (standard conditions). At confluence the medium was discarded, and the monolayer was infected with 10^8 plaque-forming units of WNV in 1 ml of DMEM (i.e., multiplicity of infection of 5) for 1 hr at 37°C. The cells were washed three times with 50 ml of phosphate-buffered saline, 20 ml of DMEM was added, and the flasks were incubated for 48 hr under standard conditions. Then cells and medium were freeze-thawed to −70°C to release intracellular virus. The medium was clarified by centrifugation at 1500 × *g* for 20 min at 4°C. The resulting pellet was sonicated in 5 ml of supernatant and centrifuged to recover remaining virus. WNV-containing supernatants were pooled and stored at −70°C. The titer was determined by serial dilution and titration on Vero cell monolayers (12).

Virus was inactivated by UV irradiation at 400 μW/cm^2 for 10 min. Inactivation was confirmed by titration as above.

Abbreviations: TGC, trophoblast giant cell(s); MHC, major histocompatibility complex; IFN-α, -β, or -γ, α, β, or γ interferon; rTNF-α, recombinant tumor necrosis factor α; WNV, West Nile virus; MEF, mouse embryo fibroblasts; EPC, ectoplacental cone; pc, postcoitum; T_c cell, cytotoxic T cell.

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IFNs and Anti-IFNs. Purified recombinant IFN-γ was a generous gift from Boehringer Ingelheim. Units stated are those given by them. Murine IFN-β (lot no. 83001), IFN-α (lot no. 83058), rabbit anti-mouse IFN-α/β antibody (lot no. 85039), and its preimmune serum (lot no. 86027) were purchased from Cytoimmune Reagents, Lee Biomolecular Laboratories (San Diego, CA). The anti-IFN-α/β antibody was found to abrogate the effect of IFN-β on a unit-for-unit basis. Recombinant tumor necrosis factor α (rTNF-α) was a gift from Ian Clark (Department of Experimental Pathology, John Curtin School of Medical Research).

Treatment of Cultures. Both C57BL/6J and (C57BL/6J × CBA/H)F_1 blastocyst and EPC preparations were allowed to hatch and adhere to the plastic coverslip for 36 hr in 1 ml of medium. At this time the following three separate treatment protocols were used.

Infection with WNV. Medium (0.5 ml) was removed, and 10^7 plaque-forming units of WNV in 0.5 ml was added to the cultures. Infected preparations were then divided into two groups: (*i*) WNV alone and (*ii*) WNV with 200 units/ml of anti-IFN-α/β antibody. Both of these groups were then undisturbed 16–20 hr before labeling.

Treatment with poly(I·C). Uninfected preparations were treated for 20 hr with poly(I·C) at 50 μg/ml. To approximate the kinetic conditions of viral infection, poly(I·C) was not washed off. This protocol produces a significant increase in class I MHC antigen expression on MEF (9).

Treatment with rTNF-α. Uninfected preparations were treated for 20 hr with rTNF-α at 100 ng/ml.

Treatment with IFN species. Five thousand units of IFN-γ or five hundred units each of IFN-α and IFN-β per ml were added to uninfected cultures of EPC and blastocyst preparations for 20 hr before labeling.

Blastocyst and EPC preparations continued to develop *in vitro* during the above treatments without obvious ill effects.

Treatment of MEF with WNV, poly(I·C), and interferons was carried out as described (9). Treatment of MEF with rTNF-α was carried out as described above.

Antibodies. Monoclonal antibodies against surface antigens K^k (clone 11-4.1; Beckton Dickinson), I-A^k (clone 11-5.2.1.9; ATCC no. TIB-94; ref. 13), and I-A^d (clone MK-D6; ATCC no. HB-3; ref. 14) were used to label MHC antigens on cells. Affinity-purified polyclonal goat anti-mouse immunoglobulin conjugated to 5-nm colloidal gold (GAMIg G5 EM grade, Janssen Pharmaceutica) was used as a second antibody.

Cell Labeling for EM. Blastocyst and EPC preparations were incubated with anti-MHC antibody, washed, and labeled with GAMIg G5 as described (6, 7). Separate (C57BL/6J × CBA/H)F_1 preparations were labeled with anti-K^k, anti-I-A^k, and anti-I-A^d as a control. C57BL/6J controls were labeled with anti-K^k. MEF were labeled in suspension. All samples were prepared for EM as described (6, 7). Methods for gold quantitation have been described in detail (15).

RESULTS

WNV Induces the *de Novo* Expression of Surface Class I MHC Antigens on Primary TGC During IFN-α-Refractory Period Equivalent to Implantation. In this study, (C57BL/6J × CBA/H)F_1 and C57BL/6J blastocysts and EPC infected with 10^7 plaque-forming units of WNV for 16–40 hr were immunogold-labeled for paternal class I and class II MHC antigens and were examined by EM for membrane-associated gold (see Table 1). In four separate experiments, paternal class I MHC antigens were readily detectable after 16-hr and 40-hr infection of (C57BL/6J × CBA/H)F_1, primary (Fig. 1) and secondary TGC, with WNV. In all experiments virus was seen in the cytoplasm (Fig. 1 *Inset*). Quantities of gold detected (15) on WNV-infected primary and secondary TGC were equivalent to those found on untreated (C57BL/6J × CBA/H)F_1 MEF and were about one-third that found on IFN-γ-treated secondary TGC (6, 7). Thus, in both cases MHC antigen induction was less than that previously found on IFN-γ-treated secondary TGC. Mock-infected (Fig. 2) and WNV-infected C57BL/6J controls showed no gold labeling. Similarly, no gold was seen on (C57BL/6J × CBA/H)F_1 preparations when anti-I-A^k or anti-I-A^d was used as the first antibody. This demonstrates that paternal class I antigens are induced *de novo* by WNV infection during the IFN-refractory period of implantation, and the apparent inhibition of MHC antigen expression may be overcome by WNV infection.

Table 1. Effect of WNV infection on MHC antigen expression of primary and secondary TGC

Treatment	Presence of surface antigens					
	Primary TGC			Secondary TGC		
	K^k	I-A^k	I-A^d	K^k	I-A^k	I-A^d
(C57BL/6J × CBA/H)F_1						
Mock-infected	−	−	−	−	−	−
WNV-infected						
16 hr	+	−	−	+	−	−
40 hr	+	−	−	+	−	−
C57BL/6J						
Mock-infected	−			−		
WNV-infected						
16 hr	−			−		
40 hr	−			−		

+, Presence of gold labeling; −, absence of gold labeling.

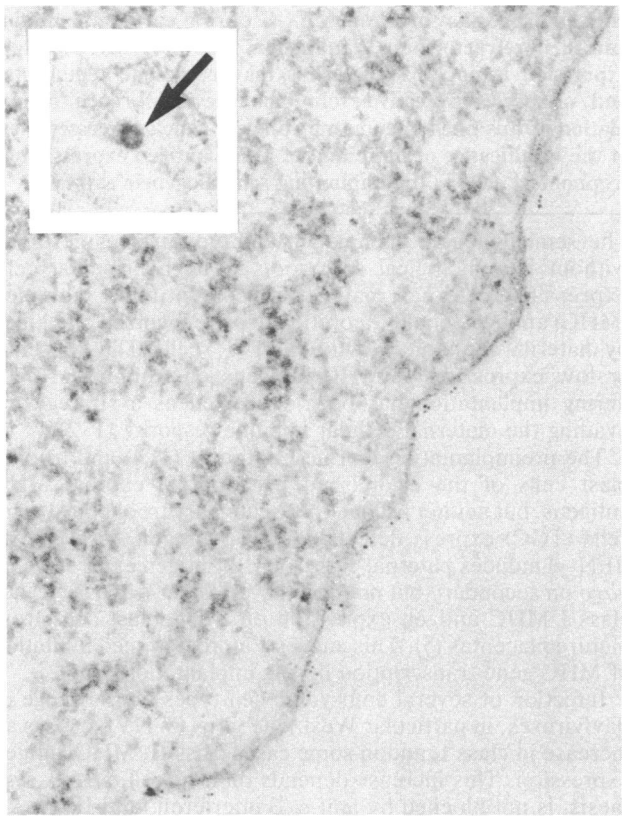

FIG. 1. Electron micrograph of primary TGC from (C57BL/6J × CBA/H)F_1 preimplantation blastocyst outgrowth infected for 16 hr with WNV and labeled with immunogold. Membrane-associated colloidal gold particles indicate the presence of paternal antigens (H-2K^k) induced by WNV infection. Virus was found in the cytoplasm of cells in all WNV-infected preparations (see *Inset*). (×76,500; *Inset*, ×45,000.)

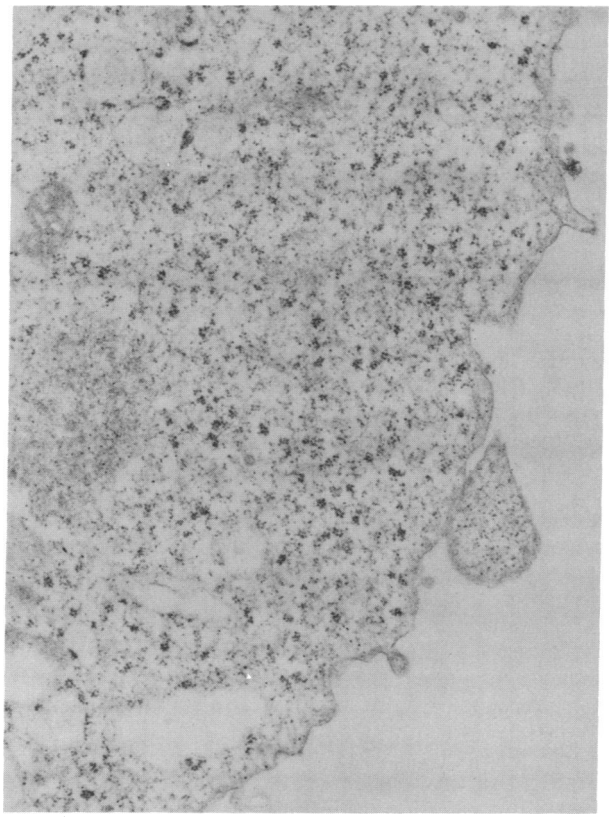

FIG. 2. Electron micrograph of primary TGC from (C57BL/6J × CBA/H)F_1 preimplantation blastocyst outgrowth mock-infected and labeled to detect the presence of paternal antigens (H-2K^k) with immunogold. Colloidal gold was not seen in any control preparations. (×18,400.)

The Induction of Paternal Class I MHC Antigens in Primary TGC Is Not Mediated by Extracellular Secretion and Feedback of Virus-Induced IFNs. WNV-induced MHC antigen expression was seen in primary TGC at day 5.5–6 pc, whereas MHC antigen expression was not inducible by IFN-α/β (virus-induced IFN) or IFN-γ (immune type IFN) before day 9 pc on either trophoblastic (ref. 7; N.J.C.K., unpublished data) or embryonic cells (8). Furthermore, virus-induced secreted IFNs are not detectable before day 7 pc in early trophoblast cells and day 8 pc in embryonic cells (16). Nevertheless, to ascertain whether the induction of MHC antigen expression was mediated by WNV-induced secreted IFN-α and/or IFN-β feeding back onto the primary TGC to induce MHC antigen expression, we conducted the following experiments. First (Table 2), purified polyclonal anti-murine IFN-α/β antibodies were added to primary TGC after 1 hr of incubation at 37°C with WNV. Mock-infected and WNV-infected primary TGC treated with "preimmune antibody" and also without antibodies were run concurrently as controls. Fifteen hours later, all primary TGC were labeled. Anti-IFN-α/β antibodies did not prevent the expression of paternal class I MHC antigens on WNV-infected primary TGC (Fig. 3), while negative controls showed no gold labeling (not shown). Second (Table 3), to ascertain whether the WNV-infected cells secreted a factor(s) that was not inactivated by anti-IFN antibodies and was able to cause either *de novo* MHC antigen expression or an increase in existing expression, the supernatants from these cultures were saved, subjected to UV-irradiation to inactivate remaining WNV, and immediately put onto fresh C57BL/6J × CBA/H)F_1 and control C57BL/6J primary TGC and MEF. This protocol leaves the MHC-increasing activity of IFN-α, -β, and -γ undiminished, while that of WNV is lost (N.J.C.K. and A.M.K., unpublished data). After incubation of primary TGC with UV-irradiated supernatants for 20 hr (40 hr in the case of MEF), expression of paternal MHC antigens was examined in comparison with primary TGC and MEF controls treated with UV-irradiated WNV-infected or mock-infected super-

Table 2. Effect of simultaneous anti-IFN-α/β treatment on MHC antigen expression of WNV-infected primary TGC

Treatment	Surface antigens on primary TGC		
	K^k	I-A^k	I-A^d
(C57BL/6J × CBA/H)F_1			
Mock-infected	−	−	−
+ anti-IFN-α/β Ab	−		
+ preimmune Ab	−		
WNV-infected			
16 hr	+	−	−
+ anti-IFN-α/β Ab	+		
+ preimmune Ab	+		
C57BL/6J			
Mock-infected	−		
+ anti-IFN-α/β Ab	−		
+ preimmune Ab	−		
WNV-infected			
16 hr	−		
+ anti-IFN-α/β Ab	−		
+ preimmune Ab	−		

+, Presence of gold labeling; −, absence of gold labeling; Ab, antibody.

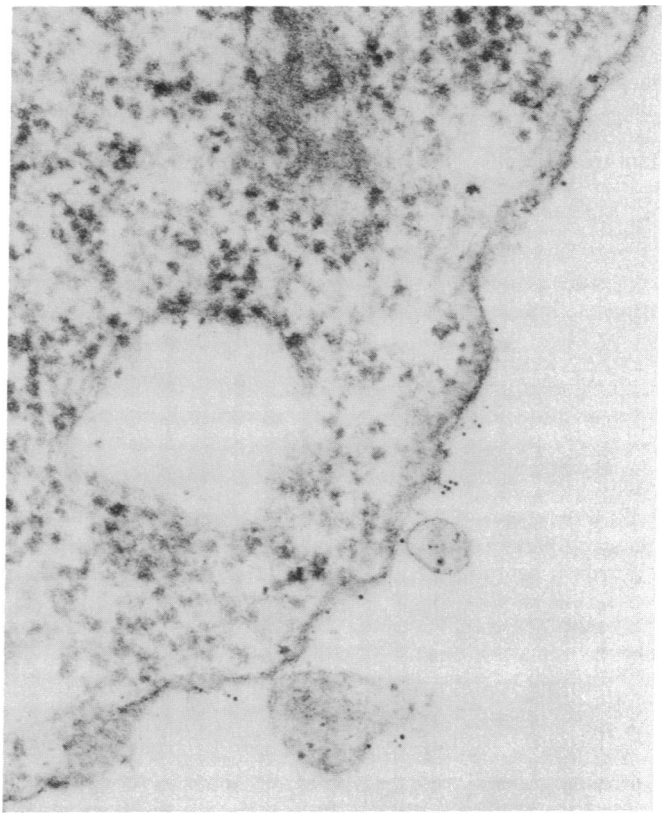

FIG. 3. Electron micrograph of primary TGC from (C57BL/6J × CBA/H)F_1 preimplantation blastocyst outgrowth infected for 16 hr with WNV in the presence of 200 units of anti-IFN-α/β antibody per ml and labeled with immunogold. Membrane-associated colloidal gold particles indicate the presence of paternal antigens (H-2K^k) induced by the WNV infection in similar quantities to those seen in Fig. 1. (×100,800.)

Table 3. Effect of UV-irradiated supernatant from WNV-infected primary TGC cultures on MHC antigen expression of primary TGC and MEF

Treatment	Presence of surface antigens					
	Primary TGC			MEF		
	K^k	$I\text{-}A^k$	$I\text{-}A^d$	K^k	$I\text{-}A^k$	$I\text{-}A^d$
$(C57BL/6J \times CBA/H)F_1$						
No treatment	−	−	−	+	−	−
Mock-infected	−	−	−	+	−	−
WNV-infected, 16 hr	+	−	−	+++	−	−
UV-irradiated SN from primary TGC cultures						
WNV-infected	−			−		
Mock-infected	−			−		
+ IFN-α/β (500 U each)	−			++	−	−
+ IFN-γ (5000 U)	−			+++	−	−
C57BL/6J						
No treatment	−			−		
Mock-infected	−			−		
WNV-infected, 6 hr	−			−		
UV-irradiated SN from primary TGC cultures						
WNV-infected	−			−		
Mock-infected	−			−		
+ IFN-α/β (500 U each)	−			−		
+ IFN-γ (5000 U)	−			−		

U, units; SN, supernatant; +, presence of gold labeling; −, absence of gold labeling; ++, increase of ≈2-fold from +; +++, increase of ≈3-fold from +.

natants and UV-irradiated mock-infected supernatants to which IFN had been added (either 500 units each of IFN-α and -β or 5000 units of IFN-γ per ml). The last control was included to make certain UV-irradiation had not destroyed or altered the supernatant medium nutrients, a situation that in itself might have prevented an MHC increase in the supernatant-treated cells. No gold labeling was found on any of the experimental primary TGC samples assayed (Fig. 4) or their controls, except on those infected with WNV, and no increase in labeling was seen in any of the MEF samples, except in WNV-infected MEF or in MEF to which IFNs had been added.

These results suggest that no detectable quantities of WNV-induced IFNs or other factor(s) are secreted. Furthermore, where anti-IFN-α/β antibody was added to WNV-infected primary TGC (Fig. 3), the presence of large amounts of IFN-α/β-neutralizing antibody makes it doubtful that small but active amounts of embryo- or TGC-secreted IFN-α and -β acted on the primary TGC to produce class I MHC antigen expression.

Does WNV Act on Primary TGC Through Intracellular Production and Activity of IFN-α or IFN-β? IFN-γ may, without being secreted, act intracellularly to increase cell surface MHC antigen expression (17), but published work to date implies that the same is not true of IFN-α and IFN-β (18, 19). Nevertheless, we attempted to ascertain whether IFN-α and -β could be induced intracellularly in primary TGC and produce MHC antigen expression without being secreted (Table 4). First, poly(I·C), which presumably mimics viral genetic material and stimulates production of IFN-α/β, was added to fresh primary TGC cultures for 20 hr before labeling. Secondly, rTNF-α, the anti-viral and MHC-increasing effects of which rely on the production of IFN-β_1 (19), was added to fresh primary TGC cultures for 20 hr before labeling. On examination, neither poly(I·C) nor rTNF-α induced MHC antigen expression on primary TGC. WNV-infected primary TGC-positive controls showed induction of class I MHC antigens, and poly(I·C)-treated, rTNF-α-treated, and WNV-infected MEF controls showed an increase in class I MHC antigen expression during this time. However, as we could not be certain that (*i*) poly(I·C) entered the primary TGC or (*ii*) functional receptors for rTNF-α were expressed on primary TGC, these results are inconclusive.

DISCUSSION

In this report we have shown that MHC antigen expression may be induced in MHC-negative, IFN-γ-refractory primary

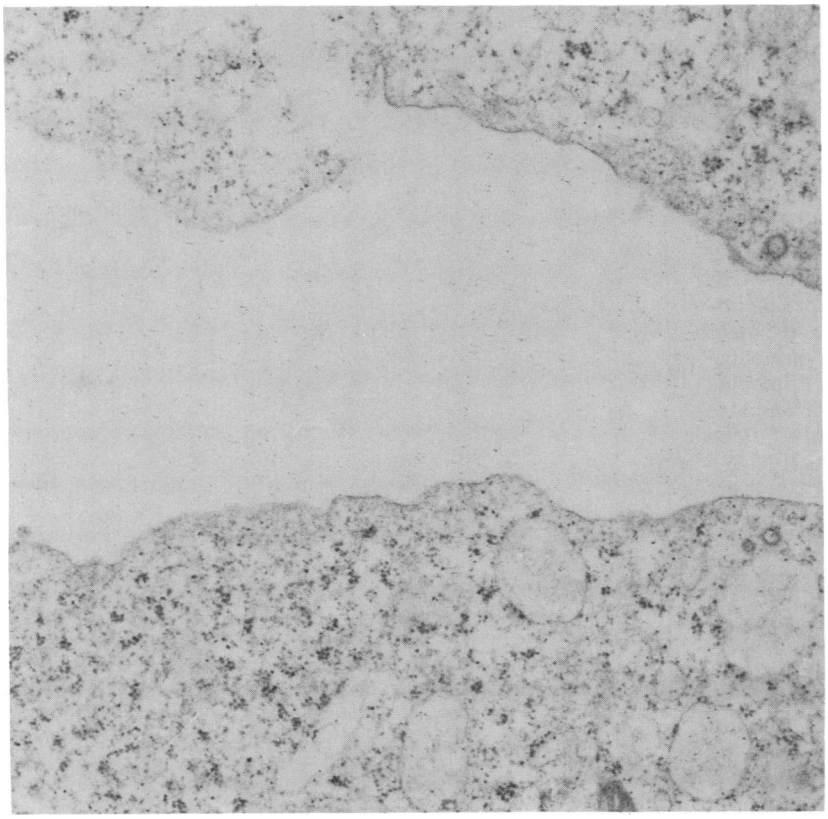

FIG. 4. Electron micrograph of primary TGC from $(C57BL/6J \times CBA/H)F_1$ preimplantation blastocyst outgrowth labeled for paternal antigens ($H\text{-}2K^k$) with immunogold after incubation for 20 hr with UV-irradiated supernatant from 16-hr WNV-infected primary TGC culture. No membrane-associated colloidal gold particles were seen and no cytoplasmic virus was observed. (×26,100.)

Table 4. Effect of poly(I·C) or TNF-α treatment on MHC antigen expression of primary TGC and MEF

Treatment	Presence of surface antigens					
	Primary TGC			MEF		
	K^k	$I\text{-}A^k$	$I\text{-}A^d$	K^k	$I\text{-}A^k$	$I\text{-}A^d$
$(C57BL/6J \times CBA/H)F_1$						
No treatment	−	−	−	+	−	−
Mock-infected	−	−	−	+	−	−
WNV-infected, 16 hr	+	−	−	+++	−	−
Poly(I·C), 20 hr	−			++		
rTNF-α, 20 hr	−			++		
C57BL/6J						
No treatment	−	−	−	−	−	−
Mock-infected	−	−	−	−	−	−
WNV-infected, 16 hr	−			−		
Poly(I·C), 20 hr	−			−		
rTNF-α, 20 hr	−			−		

+, Presence of gold labeling; −, absence of gold labeling; ++, increase of ≈2-fold from +; +++, increase of ≈3-fold from +.

TGC by infection with WNV *in vitro* during the period equivalent to initial implantation. This is unlikely to have been caused by virus-induced IFNs or other primary TGC-secreted factors, as it occurred in the presence of high concentrations of anti-IFN-α/β antibodies and was not induced by virus-inactivated supernatants from MHC antigen-induced primary TGC cultures. Primary TGC were positively identified by morphological criteria [EM and light microscopy (20)] and by fluorescent staining (light microscopy) of cytokeratin intermediate filaments typical of primary TGC (not shown) as described (6, 21).

Cells of the early embryo (inner cell mass and derivatives) were not directly examined in these experiments, but work of other investigators shows these cells to be refractory to the MHC antigen-increasing effects of IFN-α, -β, and -γ before day 8 pc (ref. 8), suggesting that the control of MHC antigen expression may be similar in both embryonic and placental precursors at this stage.

Although the quantities of induced MHC antigen in WNV-infected primary TGC were low, from a functional point of view, this level of expression is capable of inducing a significant alloimmune T_c response *in vitro* (R. V. Blanden of Division of Virology and Cellular Pathology, John Curtin School of Medical Research, personal communication). Furthermore, in other work, we have found that WNV-induced increases in MHC antigen expression are detectable within 8 hr on a variety of embryonic cells, continue for at least 96 hr, and are a feature of infection with several flaviviruses (9, 10). This may be advantageous to the adult host as the efficiency of lysis of WNV-infected target cells by WNV-immune and alloimmune T_c cells increases with time (N.J.C.K. and A.M.K., unpublished data). However, unusual induction of MHC antigen expression on cells of the embryo and placenta early in gestation, particularly if viral antigen is concurrently expressed, may theoretically result in destruction of the embryo by both virus-immune and alloimmune maternal T_c cells. This might explain the high incidence of resorption, abortion, and stillbirth in transplacental infection of embryos of several mammalian species by many flaviviruses (22), especially if infection occurs during initial implantation (23).

Although MHC antigens have been detected at low levels on preimplantation murine blastocysts (3, 4) and established placental trophoblast cells (5), attempts to demonstrate MHC antigen expression on primary TGC during initial implantation have failed. Moreover, neither the virus-induced IFN (IFN-α/β) nor immune-type IFN (IFN-γ) induces MHC antigen expression during this period, whereas later it is readily inducible by both types on placental and embryonic tissues (5–8). Hence, it has been suggested that inhibition of paternal MHC antigen expression may facilitate evasion of the maternal immune response in early implantation (2, 6, 7).

Therefore, the demonstration that suppression of paternal MHC antigen expression is not absolute during implantation is most important because manipulation of this phenomenon may finally allow a realistic experimental inquiry into whether lack of MHC antigen expression and the associated unresponsiveness to immunoregulatory factors in trophoblast cells during this period is fundamental or coincidental to the engraftment of the semiallogeneic fetus.

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