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Human parvovirus B19 VP2 empty capsids bind to human villous trophoblast cells *in vitro* via the globoside receptor

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Background: Pregnant women acutely infected with human parvovirus B19 (B19) may transmit the virus to the developing fetus. The mechanism whereby the virus interacts with the placenta is unknown. It is known that globoside receptor is required for successful infection of the target cells, which are the highly undifferentiated, actively dividing colony and burst-form units of the erythroid series. Globoside is present on trophoblast cells which have intimate contact with maternal blood, and may therefore serve as a potential route for B19 transmission into the fetal compartment.
Objectives: The purpose of this study was to determine whether B19 VP2 capsids could bind to villous trophoblast cells *in vitro* and whether globoside was involved.
Methods: Binding of B19 VP2 empty capsid to first-trimester villous trophoblast cells was assessed by multiple approaches, including ICC using either biotinylated B19 VP2 empty capsid or unlabeled B19 VP2 empty capsid. Quantification of viral binding involved I^{125}-labeled B19 VP2 empty capsid. Competition studies included excess unlabeled empty capsids or pretreatment with globoside-specific IgM antibody.
Results: Linear binding of B19 VP2 capsid to purified villous trophoblast cells *in vitro* was clearly demonstrated ($R^2 = 0.9524$). Competition studies revealed specificity of I^{125}-labeled B19 VP2 capsid binding to villous trophoblast cells when pretreatment with either 60-fold excess unlabeled B19 capsid or globoside-specific IgM antibody took place. The results illustrated B19's ability to bind in a specific manner to globoside-containing villous trophoblast cells.
Conclusion: We speculate that the globoside present on trophoblast cells may play a role in viral binding *in vivo*, which may facilitate B19 transmission across the maternal–fetal interface.

Key words PLACENTA; B19; TROPHOBLAST; GLOBOSIDE

Human parvovirus B19 (B19) was first identified in the sera of asymptomatic blood donors while screening for hepatitis B virus[12]. B19 is a non-enveloped, linear, single-stranded DNA erythrovirus within the *Parvoviridae* family[14]. The plus- and minus-sense strands of B19 DNA are encapsidated individually during virus assembly in the course of a permissive infection[13]. The 5.5 Kb genome of B19 with its terminal inverted repeats codes for two structural proteins, VP1 and VP2, and a non-structural protein, NS1[32]. The NS1 protein is an early gene product that can be expressed in permissive and in some non-permissive cell types, and is associated with cellular apoptosis[38]. Complete viral replication is limited to the highly undifferentiated, rapidly

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dividing erythroid progenitor cells of the colony-forming unit (E-CFU) and the burst-forming unit (E-BFU)[28]. The cellular receptor necessary for B19 infection is globoside, which is expressed to high levels on human erythroid cells, making the vast majority of individuals susceptible to B19 infection[6,7]. Globoside is also expressed on some non-erythroid cells, including megakaryocytes, endothelium, fetal cardiomyocytes and trophoblast cells[20,36].

B19 inhibits haematopoetic colony formation *in vitro*[28] and induces cell-cycle arrest in these cells at the G_2 phase,[27] both of which effects result in acute anemia. In an infected fetus, the greatest risk of sequelae from B19-induced anemia occurs during the first and second trimesters. This is because of the large numbers of target erythroid progenitor cells available then, coupled with the relatively immature fetal immune system[39].

The virus binds to erythroid cells via the globoside receptor[6]. Individuals lacking globoside on their red blood cells are not susceptible to B19 infection and have no detectable B19 antibodies[7]. Globoside is present on non-erythroid cell types as well, including villous syncytiotrophoblasts and extravillous and villous cytotrophoblasts[20]. Placental globoside expression appears to be highest in first-trimester villous trophoblast cells; immunohistochemical staining reveals diminished globoside levels with increasing gestational age[20]. This finding is consistent with the greater risk of poor outcome when B19 infection occurs early in pregnancy[2,3]. Trophoblast cells have intimate contact with maternal blood: villous syncytiotrophoblasts line the maternal blood spaces and extravillous cytotrophoblasts invade the maternal blood vessels[18]. Both sites offer potential routes for vertical transmission of B19 when a woman becomes infected during her pregnancy.

The mechanism(s) for vertical transmission of B19 are not known. It is unclear whether virus can interact directly with trophoblast cells present in the maternal–fetal interface to facilitate transmission. The objective of this study was to determine whether a baculovirus-expressed B19 VP2 empty capsid could bind first-trimester villous trophoblast cells *in vitro* and whether this binding involved the globoside receptor.

MATERIALS AND METHODS

Procurement of tissue from first-trimester placentas

Permission to obtain anonymous first-trimester placentas for the purpose of isolating primary villous trophoblast cells from elective pregnancy terminations was granted by the Magee-Women's Hospital institutional review board. Placentas from pregnancies being terminated because of suspected infection were not included in this evaluation.

Isolation and characterization of placental villous trophoblast cells

Villous trophoblast cells (VTB) were isolated from first-trimester placentas using a slightly modified procedure first described by Kliman *et al.*[23] Briefly, a 4–10 g sample of placental tissue was minced and digested and the resulting cells strained before separation on a Percoll (Sigma, St Louis, MO) gradient. In some preparations, cells were additionally treated with dispase (Collaborative Biomedical Products, Bedford, MA) to reduce the amount of extracellular matrix present in the preparation and thus to enhance the cell yield. Cells in a density of 1.053–1.060 g/ml were collected off the gradient after centrifugation at 400 × g for 20 min at room temperarure (RT), and used in subsequent experiments. Leukocytes were selectively depleted from the cell preparation as previously described[16]. The resultant leuko-depleted, trophoblast-enriched placental cell preparations were characterized for purity using ICC analysis individually for cytokeratin, globoside, CD45 and CD68 as described in Table 1. At least 200 cells per slide were counted among several fields at × 200 to calculate the percentage of immunoreactive cells present within the VTB preparations.

VTB binding studies using biotinylated B19 VP2 empty capsids

Freshly isolated first-trimester VTB (2.5×10^5) were incubated in the presence or absence of 15 μg/ml biotin-labeled B19 VP2 empty capsid

Table 1 Description of the Mab and ICC conditions used to characterize preparations of enriched VTB

Antigen	*Cell/virus target*	*Mab clone*	*Mab source*	*Mab diln / inc cond.*
Pancyto	Cytotrophoblast	AE1/AE3; IgG	DAKO	1 : 100/30 min, RT
βHCG	Syncytio	None; IgG	BioGenex	1 : 200/30 min, RT
Globoside	Trophoblast	AME-2; IgM	NRC*	1 : 200/ON, 4°C
CD45	Lymphocyte	2B11 + PD7/26; IgG	DAKO	1 : 100/30 min, RT
CD68	Macrophage	KP1; IgG	DAKO	1 : 400/30 min, RT

Mab, monoclonal antibody; ICC, immunocytochemistry; diln, dilution; inc cond, incubation conditions; pancyto, pancytokeratins; syncytio, syncytiotrophoblast; none, none available; RT, room temperature; NRC*, Netherlands Red Cross (50); ON, overnight

(Biotrin International, Dublin, Ireland) for 2 h at 4°C with constant rocking in 500 μl of capsid-binding medium, i.e. Iscove's Modified Dulbecco's Medium (IMDM) (Gibco BRL, Grand Island, NY) supplemented with 1% fetal bovine serum (Gibco BRL), 1% (v/v) penicillin–streptomycin (Cellgro, Gibco Brand Cell Culture, Invitrogen, Grand Island, New York) and 25 mol/l HEPES (Gibco BRL). IMDM is commonly used when infecting cells *in vitro* with B19[31,33,34]. VTB were centrifuged at 520 $\times$ g for 10 min at 4°C. Cell pellets were washed once in ice-cold IMDM before making cytospin preparations of 2.5 $\times 10^5$ cells per slide. Cytospins were fixed in ice-cold acetone (4 min), and stored at 20°C until analyzed, when they were warmed to RT, rehydrated through an ethanol series (100%–70%, 1 min each) into PBS, treated with protein-blocking agent (30 min, RT) and probed with 20 μg/ml streptavidin-labeled FITC for 30 min at RT (Vector Laboratories, Burlingame, CA). Mounting medium containing propidium iodide (Vector Laboratories, Birlingame, CA) was used to visualize the cells.

VTB-binding studies using unlabeled B19 VP2 empty capsids

Freshly isolated first-trimester VTB (2.5 $\times$ 10^5) were incubated in the presence or absence of 15 μg/ml unlabeled B19 VP2 empty capsid in an identical manner to that described for biotinylated B19 VP2 empty capsids. Virus binding to VTB was visualized using B19-specific IgG Mab that recognizes the VP2 capsid protein (clone 5215D, Chemicon International, Temecula, CA) as previously described[18].

I^{125}-labeling of B19 VP2 empty capsids

B19 VP2 empty capsids were visualized before iodination, using transmission electron microscopy (Center for Biologic Imaging, University of Pittsburgh, PA) to confirm their predicted icosahedral shape and size (18–24 nm) expected for intact assembled B19 virions[1,9]. These self-assembled, non-infectious B19 VP2 empty capsids were generated using a baculovirus expression vector (Biotrin International). B19 VP2 empty capsids (25 μg/reaction) were labeled with I^{125} using chloramine-T as described previously[17] at the Iodination Core Facility, University of Pittsburgh, PA. The reaction was terminated by dilution in $NaP0_4$ buffer and separated on a Sephadex column (Pharmacia, Pfizer, New London, Connecticut). The average specific activity of a given preparation was 13 μCi/μg capsid protein, ranging from 8.6 to 19.4 μCi/μg. The preparations of I^{125}-labeled B19 VP2 empty capsid were always used in experiments within 2 weeks of the labeling reaction.

Binding of I^{125}-labeled B19 VP2 empty capsid to VTB *in vitro*

VTB (2.5 $\times$ 10^5) were incubated at 4°C for 1 h with increasing amounts (0.05 μg to 2.0 μg) of I^{125}-labeled B19 VP2 empty capsid in a total of 500 μl binding medium, consisting of PBS, pH 7.4, with 1% BSA. The VTB-B19 capsid mixture was centrifuged at 1000 $\times$ g for 5 min at 4°C. The resulting pellets were washed three times in 1 ml binding medium at 4°C and resuspended in 1 ml fresh binding medium. The radioactive counts per min (cpm) were measured using a gamma counter. Each experiment con-

tained a series of no-cell controls, consisting of equal volumes of I^{125}-labeled capsids added to tubes without cells, which was used to estimate the non-specific binding. All data points were run in triplicate, with the experiment performed three times. Because each experiment used a different batch of iodinated B19 VP2 capsid preparation, averages could not be calculated and error bars could not be included.

To address binding specificity, we performed competition studies using either excess unlabeled B19 VP2 empty capsid, or pretreatment of VTB with globoside-specific IgM monoclonal antibody. The excess cold capsid competition studies were carried out using VTB (2.5×10^4) incubated in binding medium at 4°C for 1 h with constant rocking, either alone or with 30-fold or 60-fold excess unlabeled B19 VP2 empty capsid. Subsequently the VTB–capsid mixtures were incubated at 4°C for 1 h with 750 ng I^{125}-labeled B19 VP2 empty capsid. VTB–capsid mixtures were centrifuged at 1000 × g for 5 min at 4°C, washed twice in 1 ml cold binding medium and resuspended in 1 ml fresh binding medium. The radioactive cpm was measured using a gamma counter. Each experiment contained a no-cell control, consisting of 750 ng I^{125}-labeled capsids without cells, which was used to estimate non-specific binding. All data points were run in triplicate and the experiment was run twice with 30X and 60X excess cold capsid and a third time with only 60X excess.

The competition experiment using the globoside-specific IgM antibody pretreatment of VTB (2.5×10^5) was conducted in binding medium incubated overnight at 4°C with constant rocking without IgM antibody pretreatment (positive control), with 100 ng of globoside-specific IgM murine monoclonal antibody, or with an equal concentration of a non-relevant IgM murine monoclonal antibody (negative control). After overnight incubation, 1.5 μg I^{125}-labeled B19 VP2 empty capsids were added and incubated at 4°C for an additional 1 h with constant rocking. Cells were pelleted at 1000 × g for 5 min, washed twice and centrifuged again at 1000 × g for 5 min. The final cell pellet was resuspended in 1 ml cold binding medium and the radioactive cpm per tube measured. All data points were analyzed in triplicate, and the competition experiment was performed twice.

Statistical analysis

Differences between treatment means were analyzed using unpaired T-test (InStat for MacIntosh, 1993 Graph Pad Software Version 2.01). For competition experiments, data was log transformed and Kruskal-Wallis Nonparametric ANOVA test and Dunn's Multiple Comparison's post-test were applied. A "p" value of < 0.01 was considered significant.

RESULTS

Characterization of freshly isolated first-trimester VTB

The vast majority (93–100%) of cells isolated from first-trimester placentas (9–13 weeks' gestation) ($n = 8$) were cytotrophoblast in nature, staining positive for pancytokeratins (data not shown). In contrast, contaminating leukocytes (CD45) or macrophages (CD68) constituted 0.0–5.0% and 0.0–7.0% of the total cells, respectively (data not shown). Most purified cells (53–79%) also expressed globoside[20].

B19 VP2 empty capsid-induced VTB aggregation

B19 virus is known to induce red blood cell aggregation via the globoside receptor[5]. First-trimester VTB incubated in the presence of B19 VP2 empty capsid at room temperature for 1 h demonstrated this property also, compared with VTB incubated in diluent alone. Figure 1 illustrates a typical cell aggregation pattern seen when 2.5×10^5 VTB were incubated in the presence or absence of 15 μg B19 VP2 empty capsid. This agglutination reaction occurred rapidly after the addition of virus capsid to VTB.

B19 VP2 empty capsid-bound first-trimester VTB *in vitro*

The ability of B19 VP2 empty capsid to bind first-trimester VTB was assessed by several approaches,

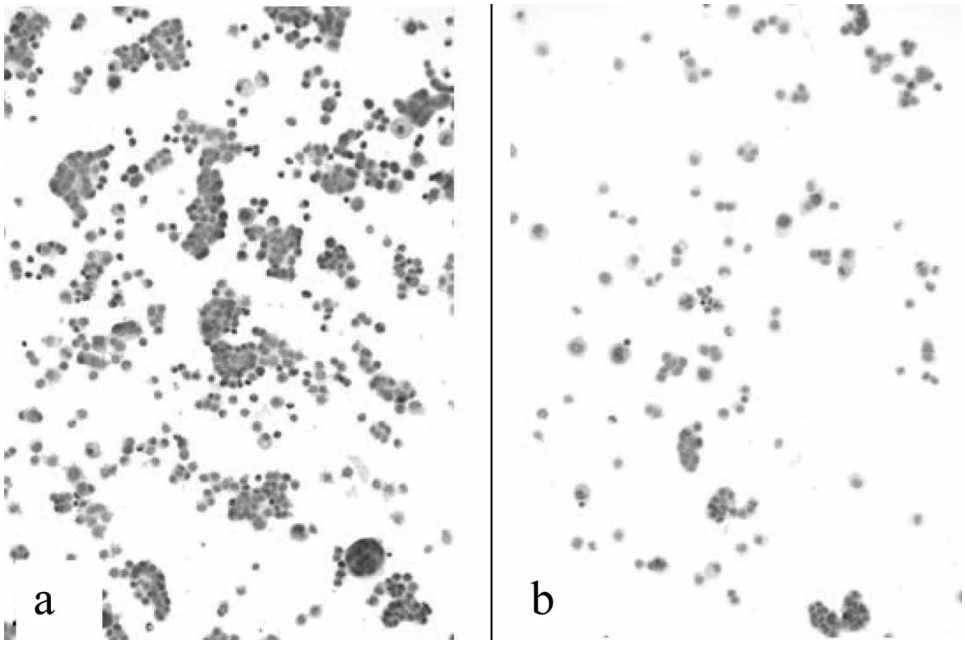

Figure 1 B19 VP2 empty capsid-induced VTB aggregation. Equal numbers of VTB (2.5×10^5) were incubated with (a) or without (b) 15 μg/ml unlabeled B19 VP2 empty capsid before cytospin preparation and hematoxylyn counterstaining

which included ICC techniques involving either biotinylated B19 VP2 empty capsid or unlabeled B19 VP2 empty capsid. Quantification of viral binding entailed I^{125}-labeled B19 VP2 empty capsid.

A streptavidin-labeled FITC probe (Vector Laboratories) was used to visualize directly biotinylated B19 VP2 capsid binding to VTB. Biotinylated B19 VP2 empty capsid (15 μg) bound to the majority of VTB was distributed over the slide, either as individual cells or as cell aggregates (Figure 2a). The bright green staining was present on the surface of the cells; only the red staining, due to the propidium iodine counterstain, was observed on VTB in the absence of B19 VP2 empty capsid (Figure 2b).

A similar pattern of VTB staining as a result of capsid binding was seen when applying unlabeled B19 VP2 empty capsid, and antigen was visualized using a B19-specific IgG Mab recognizing the VP2 capsid antigen as previously described[18]. VTB staining was absent when either B19 VP2 empty capsid was excluded from the experiment or when a non-immune serum was used in place of the B19-specific VP2 IgG Mab (data not shown).

Quantification of I^{125}-labeled B19 VP2 capsid bound to first-trimester VTB

Figure 3 illustrates the binding kinetics of B19 VP2 empty capsid with VTB. I^{125}-labeled B19 VP2 empty capsid bound VTB (2.5×10^5) *in vitro* in a linear manner ($R^2 = 0.9524$); however, saturable binding was not consistently achieved. Similar kinetics were seen regardless of whether the binding was allowed to proceed for 1 h or 2 h at 4°C (data not shown). Non-specific binding (Ficoll hypaque purified leukocytes) represented approximately 10% of the total binding at the highest amount of iodinated B19 VP2 empty capsid used (2 μg) (data not shown).

Binding of I^{125}-labeled B19 VP2 empty capsid to first-trimester VTB was reduced in the presence of 60-fold excess unlabeled B19 VP2 empty capsid

Figure 4 illustrates the decrease in I^{125}-CPM associated with VTB pretreated with 60-fold (60X) excess unlabeled B19 VP2 empty capsid compared with VTB alone without pretreatment. Because of the limited availability of B19 VP2 empty capsid, competition studies were

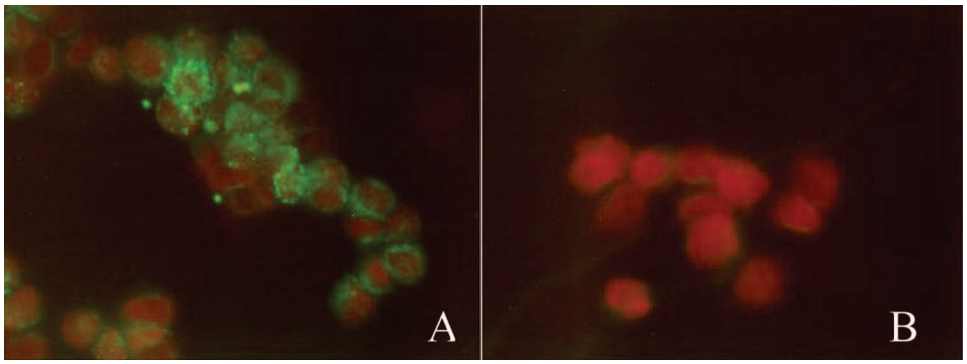

Figure 2 Evidence for B19 VP2 empty capsid bound to VTB. Biotinylated B19 VP2 empty capsids (15 μg/ml) were added to freshly isolated VTB. (a) Capsid binding was detected directly using streptavidin-labeled FITC (green). (b) Cells without added capsid revealed propidium iodine counterstain only (red)

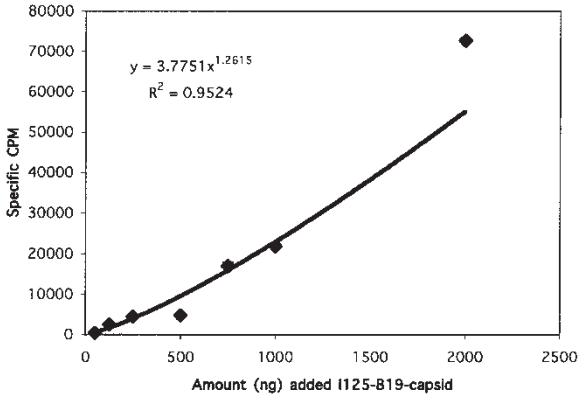

Figure 3 Binding of I^{125}-labeled B19 VP2 empty capsid to VTB *in vitro*. Increasing amounts (50 ng–2 μg) of I^{125}-labeled B19 VP2 empty capsid were added to 2.5×10^5 VTB per reaction. Cell-specific binding was defined as total counts per minute (cpm) less the non-specific cpm present in the no-cell control. Data points were analyzed by linear regression and the correlation coefficient ($R^2 = 0.9524$) was calculated

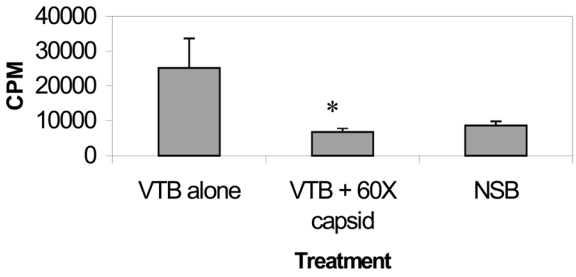

Figure 4 Effect of excess unlabeled B19 VP2 empty capsid on binding of I^{125}-labeled B19 VP2 empty capsid to VTB. I^{125}-labeled B19 VP2 empty capsid (750 ng) was added to 2.5×10^4 VTB (VTB alone), and this binding was competed for using 60-fold excess unlabeled B19 VP2 empty capsid (VTB + 60X capsid). Differences between the calculated means of VTB alone and that of the 60-fold excess unlabeled capsid competition experiment were statistically different ($p < 0.01$). Error bars represent the standard deviation of the means

carried out using 10-fold fewer cells (2×10^4) compared with the original quantitative binding studies using 2×10^5 VTB illustrated in Figure 3. VTB (2×10^4) pre-incubated alone or with 60-fold excess of unlabeled B19 VP2 empty capsid were then combined with 750 ng I^{125}-labeled B19 VP2 empty capsid. All data points were run in triplicate within an experiment. The experiment was repeated three times, twice with 30X and 60X excess unlabeled capsid, and a third time with 60X excess only. Figure 4 illustrates the mean cpm values. The one-way ANOVA demonstrated a significant difference between the mean of the VTB alone (25 162 + 8531 SD) compared with that of the VTB + 60X excess unlabeled capsid (6681 + 1116 SD) at $p = 0.0078$. Figure 4 also illustrates the effectiveness of the 60X excess unlabeled capsid competition, as these levels were similar to the mean of the non-specific binding control (8580 + 1192 SD).

Pretreatment of first trimester VTB with a globoside-specific IgM antibody resulted in reduced I^{125}-labeled B19 VP2 capsid binding

Figure 5 illustrates the level of specific binding associated with 2.5×10^5 VTB after treatment

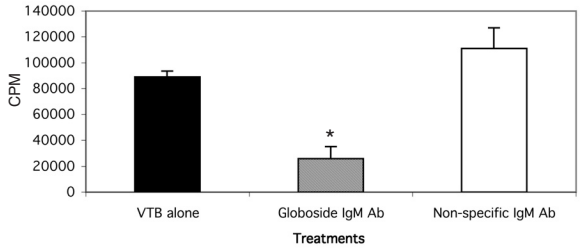

Figure 5 Effect of globoside antibody pretreatment on VTB binding of I^{125}-labeled B19 VP2 empty capsid. Equal numbers (2.5×10^5) of VTB were either untreated (black bar), or pretreated with 10 μg globoside IgM Mab (hatched bar), or with 10 μg non-specific IgM Mab (white bar) before incubation with 1.5 μg I^{125}-labeled B19 VP2 empty capsid. Error bars represent standard deviation of the means

with 100 μg globoside-specific IgM antibody with 2 μg I^{125}-labeled B19 VP2 capsid, compared with VTB without antibody pretreatment or VTB pretreatment with a non-specific IgM antibody. The difference in specific binding was highly significant ($p = 0.0004$). The level of counts associated with the no-cell control was less than 10% of the specific binding (data not shown).

DISCUSSION

The data presented here provide the first *in vitro* evidence for binding of human parvovirus B19 to placental villous cytotrophoblast cells via the globoside receptor. There is precedent for B19 binding to globoside receptors on other non-erythroid cell types, including 293 cells, HeLa cells, human umbilical vein endothelial cells and normal human lung fibroblasts. In the latter study using fibroblast cells, the investigators point out that the level of globoside expression did not correlate with efficiency of binding, and they postulate that although globoside is necessary for B19 binding, it may not be sufficient for viral entry into cells[37].

Other investigators suggest that multiple glycosphingolipids determine B19 tissue tropism[11]. We have shown previously that multiple glycolipids, including globoside, are present on villous syncytiotrophoblasts and cytotrophoblasts[20]. These related glycolipids may act as co-receptors for B19 binding to trophoblast cells.

This study further served to emphasize the success of substituting baculovirus-expressed B19 VP2 empty capsid for infectious virus, being similar in size, shape and immunoreactivity to the native virus, which is available in very limited quantities[1,9]. These recombinant B19 capsids have been shown to be similar to the native B19 virion as well, in their ability to inhibit fetal haematopoetic colony formation *in vitro*[24]. We also observed the phenomenon of villous trophoblast cell aggregation that was induced by B19 VP2 empty capsid addition, an event described by Brown *et al.*[5] for red blood cell hemagglutination in the presence of B19 capsids.

It should be noted that the binding studies described here used primary villous cytotrophoblast cells, and not villous syncytiotrophoblast cells. For the purposes of assessing viral binding, villous cytotrophoblasts were easier to isolate and enumerate compared with syncytiotrophoblast cells, and did not require culturing before experimentation. The inclusion of syncytiotrophoblasts would be much more critical in a study of viral transport across the placental barrier as opposed to assessment of viral binding. Therefore, for the purposes of this investigation, villous cytotrophoblasts were an acceptable model for viral binding. These cells contain the membrane-bound globoside receptor and are the cells whose membranes fuse to form the villous syncytiotrophoblast layer of the placenta.

The results of the competition studies suggested that viral binding to villous trophoblast cells was specific; significant reduction in viral binding was observed after pretreatment either with 60-fold excess unlabeled capsid or with globoside-specific IgM. Although the *in vitro* B19 capsid-binding to trophoblast cells was linear ($R^2 = 0.9524$), we were unable consistently to demonstrate saturable binding kinetics under the incubation conditions in our study. The observation argues against a specific receptor-mediated binding, but we believe this may be due to the limited amount of labeled B19 VP2 empty capsid used in each of the experiments as well as the natural variation in globoside content that exists between individual

placentas. We speculate that at higher virus capsid concentrations binding saturation might have been demonstrated more consistently, as described by Brown *et al.*[6] Alternative explanations might include virus use of additional receptors for binding, present on VTB. These receptors may include other glycosphingolipids known to be present on VTB[20], or a second unrelated co-receptor yet to be identified. However, it is clear from the data presented here that B19 capsids do bind to placental trophoblast cells. This is an important observation in the effort to begin to understand how B19 virions interact at the maternal–fetal interface of the placenta.

Although we have suggested a role for globoside receptor in viral binding to trophoblast cells, our data do not address B19 entry into cells. The question still remains: how does B19 virus cross the placental barrier? We are currently investigating the possibility that transplacental transfer occurs via B19-induced trophoblast cell damage and hence a breakdown of the placental barrier. B19-induced cellular apoptosis has been described in both permissive and non-permissive cell types[10,25,29,38]. Damage to the trophoblast cell layer surrounding the placenta would compromise the integrity or function of the placenta, particularly in the first trimester when active reconstruction of the placental syncytiotrophoblast barrier is occurring[30].

We have recently described an increase in the number of apoptotic syncytiotrophoblast and cytotrophoblast cells within B19-infected placentas isolated from pregnancies with poor fetal outcome, compared with B19-infected placentas isolated from pregnancies with good fetal outcome or from age-matched, uninfected control placentas[19]. The higher apoptotic indices seen in these cell types were visualized using the M30 CytoDeath antibody (Roche Molecular, Indianapolis, IN), which specifically detects apoptosis-induced cleavage of cytokeratin 18[8,21].

Other means of B19 transfer across the placenta that warrant investigation include receptor-mediated endocytosis[11,20], through the F_C receptor present on syncytiotrophoblast cells[4] or, due to its small size, passive diffusion using cell channels or pores within trophoblasts[15,22].

In summary, the results presented here have documented B19-binding to villous trophoblast cells, key cell types located at the maternal–fetal interface. A much better appreciation of how B19, a virus that is present in blood of infected individuals, interacts with the trophoblast cell is needed before we can understand the mechanism(s) by which B19 gains access to the fetal compartment. Ultimately, knowing how the virus crosses the placenta will allow us to develop approaches that may inhibit this damaging process during pregnancy.

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