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Failure of Neutralizing gp120 Monoclonal Antibodies to Prevent HIV Infection of Choriocarcinoma-Derived Trophoblasts

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Abstract
Although placental trophoblasts, the only fetal cells in direct contact with infectious maternal blood, can be infected with HIV, the precise cause for the low transmission rate of virus across the placental barrier is unknown. One of the most common conjectures is that maternal anti-HIV antibodies (Abs) contribute to the protection of the fetus. This hypothesis has been tested in vitro by infecting the CD4-negative placental trophoblast line, BeWo, with HIV-1_{IIIB} in the presence of serial dilutions of neutralizing monoclonal Abs against the V3 loop (No. 694) or CD4-binding conformational domain (No. 588). The results, based on measurement of p24 production from virus-exposed cells, reveal that the titers of Abs, adequate in preventing the infection of control MT-4 T lymphocytes, were less effective in protecting trophoblasts. Furthermore, PCR analysis of HIV DNA formed after a single round of infection has shown no significant decrease in the number of viral copies in Ab-protected BeWo cells. An anti-HIV serum from a pregnant woman did also have no effect. Although our in vitro observations do not necessarily apply to the in vivo situation, the results suggest that the humoral immune response sustained by neutralizing Abs may be able to protect T lymphocytes, but not placental trophoblasts. The findings are consistent with recent clinical studies demonstrating a lack of correlation between the presence of neutralizing anti-HIV Abs in pregnant women and HIV transmission in utero.

The transmission of HIV in utero is considered to account for a significant proportion of pediatric AIDS. The mechanism of vertical HIV transmission across the placental barrier is still poorly understood. It is not known why only a small proportion of babies born to HIV-positive mothers is infected during pregnancy. The relatively low incidence of transmission has been generally attributed to several factors: maternal virus burden, virus tropism, virus variability, pregnancy factors and humoral immune response [1, 4]. The observation that the majority of HIV-infected pregnant women are nontransmitters led to the hypothesis that neutralizing antibodies (Abs) can be effective in preventing viral spread. Several clinical studies have been undertaken during the last few years to establish a link between the presence of a defined type of anti-HIV Ab and mother-to-child viral transmission. Earlier investigations appeared to provide evidence that Abs against select epitopes of the HIV envelope protein gp120 may protect the fetus against HIV infection [18, 21, 43]. It has been proposed that mothers who have high-affinity Abs directed toward gp120 are less likely to transmit HIV to their offspring [46, 50]. However, the results of more recent studies have indicated that vertical transmission of HIV occurred despite the presence of maternal Abs [17, 23, 26], and elevated titers of anti-HIV Abs were not correlated with a reduced risk of HIV infection [41, 42, 45].

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Furthermore, higher titers of of specific IgG Abs against the V3 loop or gp41 of HIV appeared to enhance maternal transmission [32, 36]. It has been shown that, depending on the variation in the viral envelope, the monoclonal Ab against the V3 loop can either neutralize or enhance the infectious capacity of the virus [29]. A recent study concluded that low titers of neutralizing Ab were not associated with an increased risk of HIV transmission to infants [25]. The most recent studies indicate that the lack of protection in vitro can be reproduced in in vivo experiments using animal models [47].

In view of such conflicting reports, sometimes originating from the same research group but at different time points, it is difficult to comprehend whether maternal Abs against the viral envelope promote or inhibit HIV transmission. In earlier studies, we established an in vitro model of HIV transmission in utero that allowed us to test how various pregnancy hormones and trophoblast-secreted substances may affect viral transmission. As a result, we were able to identify the anti-HIV activity of chorionic gonadotropin, steroid pregnancy hormones, placental interferon, etc. [6–13]. In this study, we examined the role of maternal anti-HIV Abs in vitro by inoculating choriocarcinoma-derived BeWo trophoblasts with HIV-1 in the presence of monoclonal neutralizing Abs against either the V3 loop or CD4-binding domain of the viral envelope.

Materials and Methods

Monoclonal Antibodies and Serum

Two human monoclonal Abs, No. 588 (raised against the CD4-binding domain) and No. 694 (against the V3 loop of HIV envelope glycoprotein), were produced by fusing a mouse myeloma cell line with IgG1-secreting B lymphocytes from HIV-infected individuals [22, 27]. In some of the experiments, serum from an HIV-positive pregnant woman was used. This serum is reactive with all HIV structural proteins by Western blot and, at a dilution of 1:1024, inhibits HIV_{IIIB} infection in MT-4 T cell assays.

Neutralization Assay

Choriocarcinoma-derived trophoblast cells, BeWo (ATCC), were plated (10^4 cells/well) in 96-well plates and grown for 24 h in RPMI-1640 medium with 10% heat-inactivated FCS, *L*-glutamine, penicillin and streptomycin. CD4-positive MT-4 lymphocytes (AIDS Research and Reagents Reference Program, NIH, Rockville, Md., USA) were used as control target cells and were grown in similar conditions (10^5 cells/well). Serial semi-log dilutions of antiviral Abs, i.e. No. 588 against the CD4-binding domain and No. 694 against the V3 loop (range 0.1–10 μg/ml), were added to BeWo trophoblasts or MT-4 lymphocytes for 0.5 h at room temperature, followed by addition of 8×10^9 viral particles from IIIB-producing H9 lymphocytes at 37°C. In prior studies, it has been established that at least 1 out of 100 virions is infectious [5]. At the end of a 3-hour incubation, the inoculated cells were thoroughly washed from residual virus and used either for PCR analysis of proviral DNA or were tested for viral production by p24 ELISA at defined time points. The viability of HIV-infected trophoblasts was not different from uninfected BeWo controls up to 6 months after infection.

Virus Recovery from Infected BeWo Cells

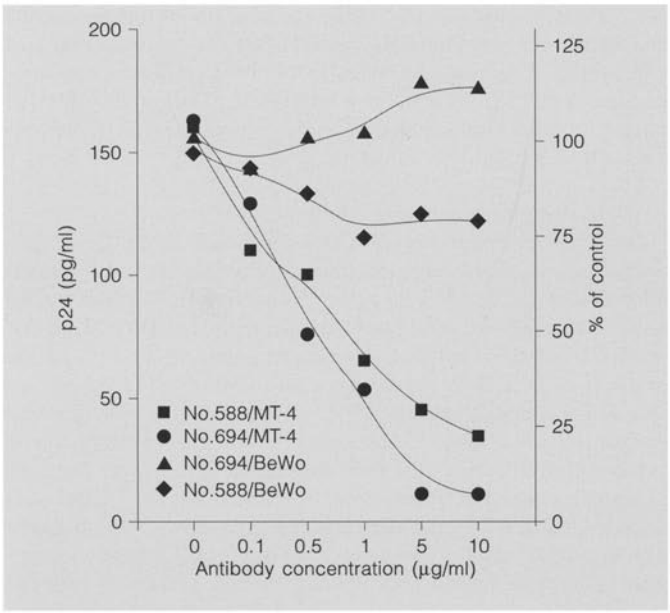
With the exception of monocytes and lymphocytes, the latency phenomenon is characteristic of most cells and tissues which are permissive to HIV. In order to assess productive HIV infection, one must rescue the virus from latently infected placental cells. In our assay, the protective effect of Abs was measured after amplification of the virus from BeWo cells by coculture with cord-blood-derived fetal MT-4 lymphocytes [9]. MT-4 lymphocytes (10^6 cells/ml) were washed twice and added to mock-infected and infected trophoblasts at day 7 after infection. Abs were not present at this time. The lymphocytes remained in contact with the trophoblasts for 3 h and were resuspended in new wells after washing three times. By the fourth day, MT-4 cells cocultured with infected BeWo cells formed multinuclear giant cells. In order to prevent the bias associated with the counting of syncytia in clump-growing MT-4 cells, productive viral infection was quantified by measuring the release of p24 HIV *gag* antigen.

Characterization of Viral Infection by ELISA

The dose response to HIV-neutralizing Abs was quantitated on days 1 and 3 after infection by measuring the amount of newly synthesized viral p24 antigen in culture supernatants of MT-4 and BeWo cells according to instructions provided by the manufacturer (HIV core p24 *gag* antigen ELISA kit; Coulter, Hialeah, Fla., USA). The amount of released HIV was quantitated by comparing the optical density with supplied p24 standards [5]. The lower limit of reliable sensitivity of this kit is 7.8 pg/ml.

Polymerase Chain Reaction and Analysis of HIV DNA

Taq polymerase extends only DNA targets; hence, the levels of newly formed proviral DNA in virus-exposed cells can serve as a reliable indicator for the blocking effect of Abs. Under the polymerase chain reaction (PCR) conditions of this study, cell-free IIIB virus, which we used to inoculate BeWo cells, did not generate any positive signal. At 3 h after infection, inoculated BeWo trophoblasts were washed and replated into new wells. After a determined period of time, BeWo cells were lysed directly in the 96-well plate by adding 20 μl of DNA extraction buffer containing 10 m*M* TRIS buffer, pH 8.3, 25 m*M* EDTA, 0.05% NP-40 and 1 mg/ml of proteinase K. After incubation at 56°C for 1 h and at 95°C for 15 min, 10 μl of lysate was transferred into plastic tubes containing 90 μl of PCR reaction mixture: 10 m*M* TRIS, pH 8.3, 50 m*M* KCl, 2.5 m*M* $MgCl_2$, 200 μ*M* of dATP, dGTP, dTTP and dCTP, 2.5 U of Taq DNA polymerase and SK68 and SK69 *env* primers. PCR was carried out in a Perkin-Elmer (Emeryville, Calif., USA) cycler for 30 cycles of 1 min at 94°C, 1 min at 61°C and 2 min at 72°C. The amplificate was then hybridized with [^{32}P]ATP-labeled oligonucleotide SK70 probe internal to the primer pairs for 1 h at 56°C and subjected to electrophoresis in an 8% acrylamide gel. The hybrid was visualized by autoradiography after overnight exposure to an X-ray film (X-Omat AR; Kodak, Rochester, N.Y., USA) at –20°C. Every amplification included, as a positive control, DNA from BeWo cells which was inoculated with HIV in the absence of Abs.



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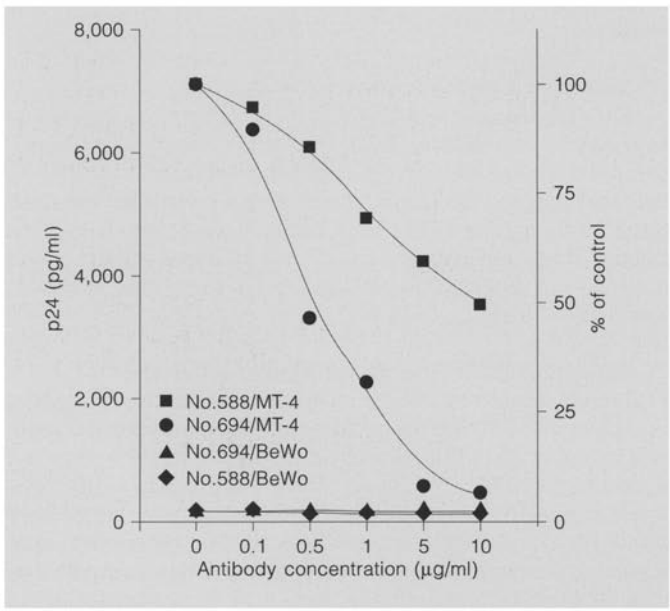

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Fig. 1. Effect of neutralizing Abs on HIV infection as measured by p24 ELISA on day 1 after infection. Abs were present during the 3-hour inoculation period. Experiments were repeated three times. Target cells were then washed from nonabsorbed HIV, and the dose effect was measured 24 h later by p24 ELISA. p24 levels are shown on the left y axis. The values on the right y axis represent the percentage scale. While there was a clear dose response with MT-4 T lymphocytes, the effect with BeWo cells was less pronounced.

Fig. 2. Effect of Abs on HIV infection as measured on day 3 after infection. Abs were present during the 3-hour inoculation period, and the dose effect was measured by p24 ELISA (left y axis). The inhibition of viral transmission is shown as percentage of the control on the right ordinate. The dose effect is clearly evident with MT-4; it cannot be determined with BeWo cells since p24 levels in trophoblast cultures decreased to the limit of detection by ELISA.

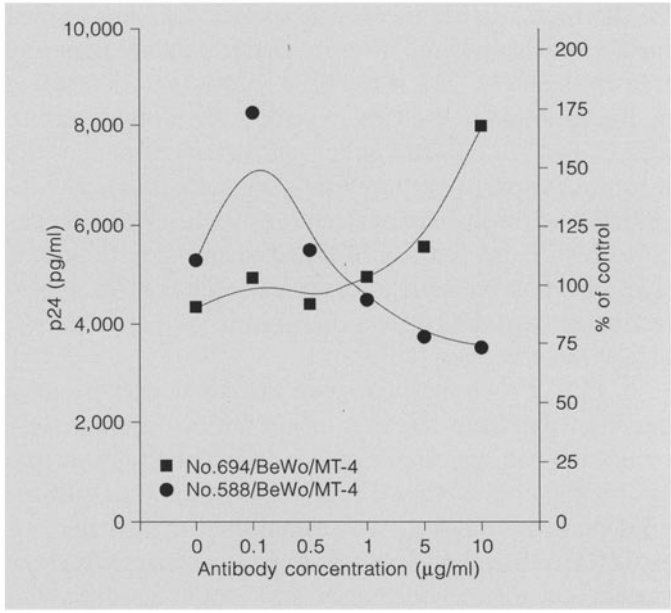

Fig. 3. Effect of Abs on HIV infection in BeWo cells as measured after rescuing the virus on day 7 after infection upon coculture of BeWo cells with uninfected MT-4 lymphocytes. No Abs were present during coculture. HIV was allowed to grow in MT-4 cells for 4 days, and the dose effect was measured by p24 ELISA (left y axis). The effect on viral infection is also shown as percentage of the control on the right ordinate. Anti-V3 loop No. 694 Ab appeared to enhance the viral output. The CD4-binding domain No. 588 Ab decreased viral production at high doses, but enhanced it at low doses.

Results

Neutralization Experiments

The experiments based on ELISA, measuring the release of p24 from virus-exposed cells, indicated that monoclonal Abs against the V3 loop of gp120 or the CD4-binding domain of HIV were effective in neutralizing the HIV_{IIIB} infection in MT-4 lymphocytes. Although the same IIIB strain of the virus was used to infect choriocarcinoma-derived BeWo trophoblasts, both Abs appeared to be ineffective in this case (fig. 1). The trophoblasts, like other CD4-negative cells, do not support active HIV replication. On two occasions, we have followed HIV-infected BeWo cells up to 6 months after infection, without observing any deterioration in viability. Thus, while in lymphocytes HIV production expands with time and results in cell killing, the viral replication in trophoblasts tends to go into latency, without harming the host cells. This phenomenon is reflected in figure 2, where HIV production has been measured at day 3 after infection. It can be seen that in MT-4 cells, both neutralizing Abs prevent-

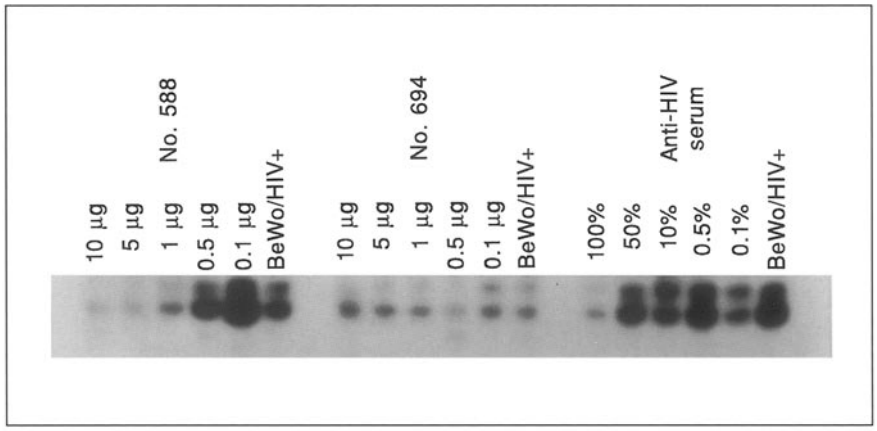

Fig. 4. HIV DNA in BeWo trophoblasts infected with IIIB strain of HIV derived from H9 lymphocytes. At 3 h after infection, BeWo cells were washed and lysed in DNA extraction buffer containing protease K. Proviral DNA formed after initial inoculation was amplified by PCR using SK68 and SK69 *env* primers. HIV-*env*-specific signals were revealed by autoradiography after hybridization with a [^{32}P]ATP-labeledoligonucleotide SK70 probe. The results with No. 588 and 694 Abs are essentially similar to the results from coculture experiments. Anti-HIV serum had no effect, except with undiluted 100% serum which was toxic to BeWo cells.

ed HIV production in a dose-dependent fashion, whereas in BeWo cells, there was no discernible effect. Due to the low output of p24 in trophoblast cultures, it is apparent that the direct ELISA measurement cannot provide satisfactory evidence for the effect of neutralizing Abs in this type of CD4-negative cells. Two independent approaches were undertaken to overcome this drawback. In the first, the virus was rescued by coculturing chronically infected BeWo cells with T lymphocytes, and in the other, the levels of proviral DNA in BeWo cells were analyzed after amplification by PCR.

Recovery of HIV from BeWo Cells by Coculture with MT-4 Lymphocytes

In these experiments, we have examined the degree of productive infection in cord-blood-derived MT-4 lymphocytes after coincubation with BeWo cells which had been infected with HIV 1 week prior to this assay in the presence of different titers of Abs (fig. 3). In controls which were not protected with Abs, there was an obvious cytopathic effect characterized by syncytium formation in MT-4 cells starting from day 4 followed by lymphocyte death after 1 week. These findings indicate that latently infected BeWo cells can be induced to release HIV upon physical contact with fetal cord blood lymphocytes. The degree of infection in experimental wells corresponding to BeWo cells protected with Abs was similar to positive controls. The number of syncytial cells, as detected visually under the microscope, appeared to be the same as in positive controls. In order to quantify this phenomenon, duplicate samples of supernatants from BeWo-exposed MT-4 cultures were examined for productive infection by p24 ELISA at day 4 after coculture. The levels of de novo synthesized p24 appeared to increase linearly, along with increasing titers of No. 694 Ab, while with No. 588, the opposite trend was observed. However, even the highest doses of No. 588 Ab used in this study failed to provide complete protection against HIV.

Measurement of HIV Infection by PCR

As early as 3 h after the entry of HIV into target cells, retroviral RNA is converted by reverse transcriptase into DNA, which can then integrate into a host genome. PCR analysis of proviral DNA, formed after a single round of infection in Ab-protected cells, can provide credible proof for the dose effect of neutralizing Abs. The results of these experiments are shown in figure 4. Higher concentrations of V3 loop Ab appeared to enhance HIV infection. The low doses of Ab against the CD4-binding domain also enhanced viral infection, but higher doses appeared to block HIV. The outcome of these experiments is in agreement with the results of coculture experiments, suggesting that HIV transmission cannot be blocked by doses of Abs effective in protecting T lymphocytes in a dose-dependent manner. Diluted anti-HIV serum from a pregnant woman showed little effect. The reduction in HIV DNA levels which was observed with undiluted (100%) serum was due to its toxicity toward BeWo cells. PCR amplification of viral supernatant containing virions did not produce any bands and served as a negative control (data not shown).

Discussion

No effective strategies for preventing maternal-infant transmission of HIV are yet available. Recent results from the ACTG 076 clinical trial and other similar studies have suggested that zidovudine may reduce the risk of vertical transmission [38]. Unless there is a bias in the sample population, the results are encouraging. Long-term-safety

results will not be available for at least 10 years, and the purported lack of deleterious effects in these clinical studies should be regarded with caution [30, 49] since no complete protection has been achieved, and zidovudine therapy may favor the spread of drug-resistant strains [20]. Thus, the therapeutic options for preventing vertical transmission are limited, and other strategies such as an administration of hyperimmune immunoglobulin or vaccine to pregnant mothers have been considered as alternatives. Accordingly, clinical trials of anti-HIV vaccines in seropositive mothers were initiated with the goal of preventing mother-to-child transmission of HIV.

Considerable efforts are presently made to understand the mechanisms which allow certain variants of virus to escape humoral immune control [1, 33]. The studies based on site substitution of the HIV envelope have shown that neutralizing Abs recognizing gp120 of susceptible HIV strains cannot abrogate the infectivity of mutant viruses [29]. Although a great deal of attention has been paid to structural and functional properties of virus variants, the target cells used in many of these studies were invariably CD4-positive T lymphocytes. However, it is well known that practically every major type of human cell can be susceptible to HIV infection. It is obvious that the success of an anti-HIV vaccine will be determined by its ability to confer protection to all types of human tissues. Our results suggest that a vaccine highly effective in protecting T lymphocytes may not be effective in preventing HIV spread to other types of human tissue. This possibility is illustrated in a model which imitates mother-to-child HIV transmission in utero. It appears that the phenotype of the virus and the type of the cell involved in such assays may deviate from the expected outcome into the opposite direction as human antibodies which were effective in vitro were not capable of neutralizing the same virus in vivo [47]. Despite the progress in anti-HIV vaccine development, very little is known about the protective effects of neutralizing Abs against the spread of HIV to the tissues outside of the blood compartment. Schwartz et al. [48] suggested recently that polyclonal Abs and soluble CD4 can block cell-mediated HIV infection in chorionic villus cultures. However, these primary cultures consisted of mesenchymal fibroblastoid cells located beneath the trophoblast layer, which constitutes the outer surface of the placenta.

In this in vitro study, we have attempted to fill this gap by investigating the protective effects of neutralizing Abs on HIV infection of trophoblasts – the only type of fetal cells in direct contact with infectious virus in maternal blood. Although Abs against gp120 demonstrated a dose-dependent protective effect with T lymphocytes, no meaningful inhibitory effect was observed when CD4-negative trophoblasts were used as target cells. Both approaches, one based on measuring productive infection by p24 ELISA, the other based on measuring the levels of proviral DNA by PCR, appear to confirm this conclusion. The apparent disparity between two types of target cells, T lymphocytes and trophoblasts, indicates that the humoral protection afforded by these neutralizing Abs is of a selective nature specific to each individual category of human tissues.

It is possible that the absence of CD4 on trophoblasts accounts for this phenomenon [31, 51]. Despite conflicting evidence [16], it is likely that alternative receptors, e.g. mannose-like lectin, may be responsible for HIV infection of trophoblasts. This receptor shares antigenic determinants with CD4 which may partly account for the limited blocking effect of No. 588 Ab directed against the CD4-binding domain [15]. The involvement of Fc-γ receptors on the surface of trophoblasts cannot be ruled out [16], but in view of the discrepancy in results between two Abs belonging to the same IgG1 isotype, it is unlikely that these ligands play a major role. The interaction of HIV with the host cell is certainly more complex than originally thought. More than a dozen alternative HIV receptors have been identified in the last few years, indicating that CD4 is not the sole receptor for the virus. We have shown earlier that the trophoblasts were resistant to a small inocula of free virus, but became permissive when exposed physically to HIV-infected leukocytes or to high doses (> 1,000 virions per cell) of infectious cell-free virus [11]. This observation is in agreement with clinical studies linking vertical HIV transmission with higher viral load [28].

According to some of the recent data, HIV transmission appears to occur around the time of delivery [44]. However, there is also convincing evidence that HIV can cross the placenta in the early months of gestation [3, 14, 24, 34, 37, 39, 40]. During the 9 months of pregnancy, the fetus is sealed from the outer world by a continuous sheet of specialized placental cells – trophoblasts. It is now established that these cells can be susceptible to HIV infection and may thus serve as prime targets and vectors of HIV [2, 9, 19, 35, 51]. The possibility that HIV may infect the fetus early in pregnancy is consistent with the planned use of an anti-HIV vaccine during a time frame which encompasses the whole duration of gestation.

Little is known as to why seropositive individuals progress to AIDS despite circulating anti-HIV Abs. The recent results of the largest 5-year vaccine trial involving 608 HIV-infected persons were disappointing as no effect

in preventing disease progression was detected. The relationship of neutralizing Abs to the likelihood of transmission in utero is also unclear. The evidence of the protective role of anti-HIV maternal Abs supported by earlier studies [18, 21, 43, 46, 50] is refuted by the results of more recent work [17, 23, 26, 41, 42, 45]. Similarly, our findings do not support the hypothesis according to which physiological levels of HIV-blocking Abs may contribute to the protection of the fetus. The results from the in vitro model of HIV transmission in utero indicate that envelope-directed Abs used in our study are not effective in preventing viral infection in trophoblasts, at least at doses that are physiologically relevant.

Active and passive immunization protocols are being developed as a means of reducing vertical transmission of HIV, and clinical trials of anti-HIV vaccines are now in progress. It is anticipated that the results of these studies may help to determine whether any specific or nonspecific maternal humoral response might reduce perinatal transmission of HIV. Although most of the current vaccine candidates directed against the envelope of HIV may block infection in the virus-lymphocyte system, this study suggests that fetus-derived placental cells will be, at best, not protected by the same type of Abs. Clinical evidence suggests that there is even a risk that certain types of anti-HIV Abs might enhance vertical HIV transmission [25, 32, 36].

It is hoped that results derived from our in vitro model may advance one step forward in finding optimally effective prophylactic measures against viral transmission in utero. Our findings imply that the current strategy in developing an anti-HIV vaccine should take into account not only the problems of viral antigenic shift and Ab affinity, but also the problem of tissue restriction. It appears that a simple switch of the target cell type can potentially diminish the effectiveness of a vaccine. Further studies are required to critically evaluate this issue.

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