False Immunohistochemical Results for Herpesviridae and Other Clusters of Differentiation Due To Biotin Intranuclear Inclusions in the Gestational Endometrium

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Received: April 30, 2013
Accepted: September 5, 2013
Published Online: September 14, 2013
DOI: 10.5455/jihp.20130905124106

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Keywords: Immunohistochemistry; false results; herpesviridae; cluster of differentiation (CD); biotin; gestational endometrium

Abstract
Objective: There are now eight known human herpes viruses (HHV) containing a double-stranded DNA genome: Herpes Simplex Virus 1 (HSV1, HHV1), Herpes Simplex Virus 2 (HSV2, HHV2), Varicella-Zoster Virus (VZV, HHV3), Epstein-Barr Virus (EBV, HHV4), Cytomegalovirus (CMV, HHV5), Herpes Virus 6 (HHV6-a, HHV6-b), Herpes Virus 7 (HHV7) and Herpes Virus 8 (HHV8, KSHV - Kaposi’s sarcoma associated herpes virus).

The highest risk of maternal-fetal HSV transmission is among women who acquire a genital infection during pregnancy. Congenital HHV6 infection occurs in 1% of births, a rate similar to that for CMV infection. Congenital HHV6-a infections are asymptomatic, whereas postnatal HHV6-b infections present with acute febrile states.

The purpose of this study was to eliminate false positive results in order to achieve an accurate pathological diagnosis for herpes virus infections to the gestational endometrium.

Methods: The biopsies of three suspected cases of gestational herpetic endometritis were submitted to immunohistochemical tests for HHV1+2, HHV5, HHV6-a, HHV6-b, HHV8 and for other clusters of differentiation (CD4, CD8, and CD20) in compliance with the ABC method protocol. Samples from the three cases under study were submitted to a real-time polymerase chain reaction (PCR).

Results: All three cases exhibited false intranuclear immunohistochemical positivity to the antibodies being used. Conversely, all three reactions tested negative with biotin inactivation. The PCR for herpesviridae, which was positive in the known herpetic controls, showed negative in all three cases.

Conclusions: Immunohistochemical investigations of the gestational endometrium (particularly in pregnancies near to term) may yield false results for several herpes viruses, as well as for other immunohistochemical reactions obtained using the ABC method without prior biotin inactivation.
such infections present without signs and symptoms of disease, or at least with a clinical picture which lacks specificity in its signs and symptoms. The common misconception that most new-born babies with a neonatal HSV infection are born to mothers with a history of genital herpes has been revised in the light of new evidence. This evidence shows that most maternal-fetal transmission derives from mothers with an undiagnosed primary genital herpes infection (HSV1 or HSV2) contracted near term [1]. Analogously, the misconception that HSV infection derives mainly from non-maternal sources has been exposed by evidence suggesting that, in more than 75% of neonatal HSV1, the infection derives from a recently acquired maternal genital infection, which is subsequently transmitted to the infant during delivery [1]. Therefore, in applying caesarean section or prophylactic acyclovir therapy, correct pre-term diagnosis of maternal herpes is a prerequisite in preventing neonatal HSV infection.

CMV was isolated by culture in 39 specimens taken from cervical or endometrial surgery in 30 out of 147 women affected by pelvic inflammatory disease (PID). HSV, by contrast, was isolated in only 5 of these 147 cases [3]. HHV6 was identified in the maternal genital tract of a significant proportion of pregnant women, and congenital infection has been occasionally reported only in small cohort studies. The HHV6 congenital infection rates are around 1% [4]. The majority of HHV6 infections occur in healthy infants, mostly caused by HHV6-b [5]. Congenital infections were asymptomatic and related in one-third of cases to HHV6-a, whereas all postnatal febrile states were due to HHV6-b.

HHV6 and HHV7 are common pathogens similar in their biology, epidemiology and clinical presentation [4]. Initially isolated from peripheral blood cells, they are widely diffused viruses that may be acquired in early childhood. Surprisingly, congenital HHV7 infection has yet to be reported in literature.

It appears clear from this general outline that diagnosing herpetic infection of the gestational endometrium may prove difficult and unreliable.

**MATERIALS AND METHODS**

Three suspected cases of gestational herpes infection were studied.

**CASE 1**

The patient is a 37-year-old para II woman. The patient and her husband had a history of rubella and she was subsequently affected by VZV. Five years previously, a left salpingectomy was performed for sactosalpinx. The following year she underwent a routine scheduled caesarian section at week 39 of pregnancy for breech presentation (a viable baby weighing 3110 g at birth).

At week 17 of the second pregnancy, she suffered from vaginosis, and serological testing revealed raised IgM-antiCMV rates compatible with the recrudescence of a previous CMV infection. Routine scheduled caesarian section was performed at week 39 (a viable baby weighing 3240 g at birth). Amniochorial membranes focally adhered to the decidua, forming a mass of 2 x 1.5 cm. The microscopic pattern exhibited polypoid endometrial glands with nuclear inclusions (Figure 1) and stromal decidualization.

**CASE 2**

A 25-year-old primiparous woman has hypersensitivity for latex and nickel. She gave vaginal birth to a viable infant at week 40. One week after delivery, she developed a fever which was unresponsive to broad-spectrum multi-antibiotic therapy. Transvaginal echography revealed the presence of intrauterine material whose histological pattern resembled that of acute endometritis, comprising nuclear inclusions in the epithelial cells. The patient in the adjacent bed had a genital HSV infection, and a clinical diagnosis was made of suspected HSV postpartum endometritis.

**CASE 3**

At week 11\textsuperscript{3} of the third pregnancy, a 40-year-old para II woman suffered a metrorrhagia and was admitted to hospital with a diagnosis of suspected miscarriage, confirmed at echography and with subsequent uterine scraping. The histological examination revealed secretory endometrium, decidua, chorionic villi and scattered cytotrophoblastic cells with nuclear inclusions.

**Immunohistochemistry**

IHC for HSV1+2, CMV, HHV6-a, HHV6-b, HHV8 and also for other clusters of differentiation (CD4, CD8, and CD20) was performed using an automated procedure (VENTANA Automated Slide Stainer) with the usual ABC immunodetection system. Near-adjacent sections of each case were also processed using a special kit (VENTANA OptiView DAB IHC Detection Kit) designed to prevent the interference of endogenous biotin on immunohistochemistry performed using the ABC method.

**DNA isolation and molecular analysis**

PCR for HSV1 and HSV2 was performed on the samples of the three cases under investigation and on three control cases which were known to be herpes positive. Four-micron thick sections obtained from paraffin-embedded tissue were placed in 1.5 ml Eppendorf tubes. DNA was submitted to EDTA-SDS / protease K treatment (Applied Biosystem, Foster City, CA, USA). It then underwent phenol-chloroform extraction, as described by Ferrante et al. [6], and was
resuspended in 35 µl of DEPC-treated RNase-free water (Bioline, London, UK). All the samples were analyzed using HSV1 and HSV2 Real-Time PCR (Expereteam, Venice), which allows the analyst to differentiate HSV1 from HSV2. The molecular analyses were performed in 25 µL volume, containing 5 µL of the template extracted DNA, 12.5 µL of Taq 2X master mix, 2.5 µL of nuclease-free water (Diagenode, Belgium), 2.5 µL of Diagenode’s HSV1 / HSV2 primers, 2.5 µL of Diagenode’s double-dye probe, employing a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, USA). Signal detection of HSV1 was in FAM, signal detection of HSV2 was in Yellow dye, while signal detection of endogenous PCR control was in Orange dye. The sample was considered non-valid if all the signals were below the threshold level.

RESULTS

In all three cases the nuclear inclusions exhibited false immunohistochemical positivity for all antibodies tested using the ABC method, that is HSV1+2 (Figure 2), CD20 (Figure 1) and CMV, HHV6-a, HHV6-b, HHV8, CD4, CD8 (Figure 3). Conversely, all the reactions turned negative when the above-mentioned VENTANA special kit was employed. Moreover, PCR for herpesviridae, positive in the known herpetic controls, was found negative in all three reported cases (Figure 4).

![Figure 1](image-url): Gestational endometrium: a number of intranuclear epithelial inclusions are visible, mimicking herpetic infection (haematoxylin-eosin, x20 for panel A and x40 for panel B). The intranuclear inclusions present false positive reactions with all antibodies employed using the usual ABC method: the figure exemplifies a strong false positive reaction for CD20 (immunohistochemistry, x20 for panel C and x40 for panel D).
Figure 2: Immunohistochemistry for HSV1+2: false positivity (without biotin inhibition, x40 for panel A) and real negativity (with biotin inhibition, x40 for panel B) are shown in the same case.

Figure 3: The intranuclear epithelial inclusions due to biotin give rise to false positive immunohistochemical results for all immunomarkers tested, that is HHV6a (x20), HHV6b (x20), HHV8 (x20), CMV (x20), CD4 (x20), and CD8 (x20).
DISCUSSION

Intranuclear biotin inclusions resembling those present in herpes simplex infected cells were observed in endometrial glands, particularly during late gestation and puerperium. These inclusions are faintly stained with haematoxylin and the nuclei present chromatin edges with clear centres which create a ground-glass appearance. These nuclei can be easily mistaken for an HSV infection [7-10]. As is well known [10], biotin is a co-enzyme active in gluconeogenesis and fatty acid biosynthesis. Endogenous biotin is also present in the kidneys, liver, pancreas, mammary glands, adipose tissue and skeletal muscle [11, 12]. To the best of our knowledge, false immunohistochemical results due to biotin in the above-mentioned organs have hitherto not been reported.

The data from our series have provided false results ascribable to nuclear biotin content. This obtains not only for all antibodies directed against herpes viruses, but also for other unrelated clusters of differentiation which underwent testing (Figure 3). It is for this reason that particular care should be exercised in ascertaining cases of gestational herpetic infection, and where possible molecular techniques such as PCR should be employed.

REFERENCES

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