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NEW METHODOLOGIES FOR THE PATHOLOGIC DIAGNOSIS OF PLACENTAL INFECTIONS - IMMUNOHISTOCHEMISTRY, IMMUNOFLUORESCENCE, *IN SITU* NUCLEIC ACID HYBRIDIZATION AND POLYMERASE CHAIN REACTION
-A Review-

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INTRODUCTION

The pathologic examinaton of the placenta for infectious agents is an important component of the clinical evaluation of the newborn. A wide variety of microorganisms can be identified in formalin-fixed and routinely stained preparations of the placenta, including viral inclusions, bacteria, and parasites. These histologic techniques are easily performed and are the most common procedures used in modern histology laboratories, and the techniques have changed little since their development in the nineteenth and twentieth centuries.

Unfortunately, the low sensitivity of routine histochemistry for identification of microbial agents in tissue sections results in underdiagnosis of many placental infections. False negative results in the placental pathology examination can lead to delayed identification and proper treatment of congenital infection, in many cases progressing to permanent sequelae and, occasionally, death.

In recent years an increasing number of laboratory techniques have been developed for the accurate detection of infections in tissues. Many were originally designed as research tools, but have now been adapted for use in the pathologic diagnosis of formalin-fixed tissues which constitute the majority of specimens available for examination. Four of these techniques, fluorochrome and enzyme-labeled immunohistochemical staining, *in situ* nucleic acid hybridization, and polymerase chain reaction, will be discussed with particular attention to their role in the diagnosis of placental infections.

METHODS OF PLACENTAL EXAMINATION

Immunohistochemistry

The use of antibodies to detect and identify infectious agents in tissue sections has proven to be a valuable adjunct in the pathologic evaluation of formalin-fixed tissues

from any sites; however, it has probably been under utilized in placental diagnosis. Immunoperoxidase techniques, using the chromogens diaminobenzidine (DAB) or 3-amino-9-ethylcarbazole (AEC), or an immunoalkaline-phosphatase system, often with the chromogen fast red, are the most common antibody-based reactions used. Immunoperoxidase staining has the advantage of producing good results in both fresh frozen and formalin-fixed tissue sections of placenta. However, alkaline phosphatase methods are not suitable for use in frozen placental tissue samples, due to the high level of endogenous alkaline phosphatase activity in this organ. Because immunohistochemistry is based upon an antigen-antibody reaction, there must be adequate preservation of the antigenic characteristics of the infectious agent to produce good results. Consequently, the possibility of false negative staining in tissue which has been overfixed in formalin or other deleterious fixatives, autolyzed, or otherwise mistreated should always be considered when the results of staining are correlated with the clinical information.

Table I

Commercially Available Monoclonal Antibodies to Infectious Agents

VIRUSES

Adenovirus	HTLV
Coronavirus	Influenza
Cytomegalovirus	Measles
Epstein Barr Virus	Mumps
Hepatitis A	Parainfluenza
Hepatitis B	Human Parvovirus
Hepatitis C	Respiratory Syncytial Virus
Hepatitis Delta	Poliovirus
Herpes Simplex	Rotavirus
HIV-1,2	Rubella
Human Papillomavirus	Varicella Zoster

BACTERIA AND FUNGI

Bordetella pertussis	Leptospira interrogans
Brucella	Mycoplasma
Candida	Neisseria gonorrhoeae
Chlamydia	Pseudomonas
Escherichia coli	Rickettsia prowazeki
Francisella tularensis	Staphylococcus aureus
Helicobacter pylori	Treponema pallidum
Legionella pneumophila	

PARASITES

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Cryptosporidium	Pneumocystis carinii
Entamoeba histolytica	Plasmodium
Echinoococcus	Toxoplasma gondii

A variety of monoclonal (Table 1) and polyclonal antibodies to microbial pathogens are currently available commercially, with additional reagents for infectious agents continually being added. The most common infections that can be diagnosed using immunohistochemistry are cytomegalovirus (CMV) (Wolber and Lloyd, 1988; Robey et al., 1988) and herpex simplex virus (HSV) (Sauerberi et al., 1985). In cases of CMV infection, antibodies to early CMV antigen may detect infected cells without demonstrable cytopathic effects, an advantage compared to in situ hybridization (Robey et al., 1988). However, in situ hybridization often produces more intense staining than immunochemistry (Wolber and Lloyd, 1988). Thus, in those cases in which diagnostic viral cytopathic effects in placental tissues cannot be readily discerned with routine hematoxylin and eosin staining, we prefer to demonstrate CMV and HSV by two complementary techniques - immunohistochemistry to identify viral antigen, and in situ nucleic acid hybridization (discussed below) to confirm the presence of viral nucleic acid.

Double-staining immunohistochemistry is a technique using two different antibodies and different colored labels, which can be used to identify both infectious agents and the specific cell types in which they are located. This technique has the advantage of definitive localization of infectious agents to immunohistochemically confirmed cell types, and is especially useful in studies of microbial pathogenesis. Mühlemann et al. (1992) recently used double-staining immunohistochemistry to localize CMV placental infection to trophoblast, and endothelial and stromal cells of the chorionic villi.

An additional role for monoclonal antibodies is for the characterization of the fetal inflammatory response to infectious agents. In cases of villitis, in which inflammatory cells are present in chorionic villi, antibodies to leukocyte surface antigens are used to characterize the cellular response. Using a panel of antibodies to leukocyte surface antigens, Schwartz et al. (1991, 1992) found that the predominant response in placentae with CMV-associated villitis was T lymphocytes, Hofbauer cells, and IgM- and IgG-secreting plasma cells. A significant finding in these placentae was the occurrence of CD45RO-positive T lymphocytes, a marker for activated and memory T cells, in gestations as early as 14 weeks. Hofbauer cells, the villus stromal macrophages of fetal origin, can also be functionally and quantitatively assessed in placental infections using antibodies to macrophage antigens (Schwartz et al., 1991) (Figure 1). This technique has also been used to study the inflammatory response in placentae with villitis of unknown etiology (Labarrere et al., 1990), who found that areas of villitis contained activated (HLA-Dr, HLA-DP, HLA-DQ reactive) macrophages, T lymphocytes of CD4 (helper) phenotype, and lack of B Cells. Identification and characterization of placental cells in the proliferation phase of the cell cycle using the monoclonal antibodies Ki-67 and PCNA (cyclin) has recently been used to study congenital viral infections (Schwartz et al., manuscript in submission) (Figure 2).

Immunofluorescence

The detection of microbial antigens in clinical specimens by immunofluorescence has been used predominantly to demonstrate viral infections, including influenza and parainfluenza viruses, rubella, herpes viruses, mumps, poliovirus, rabies, respiratory syncytial virus, and some togaviruses (Gardner, 1977). Both direct and indirect tests can be used - direct fluorescence using an antibody tagged with the fluorochrome label, and indirect fluorescence using first an unlabeled microbe-specific primary antibody followed by a fluorochrome-tagged antibody to the primary antibody. Double-labeling
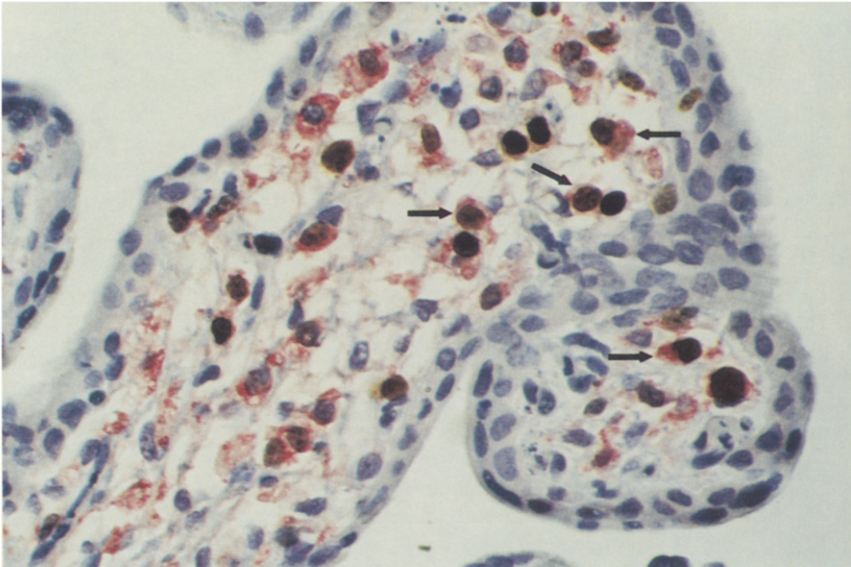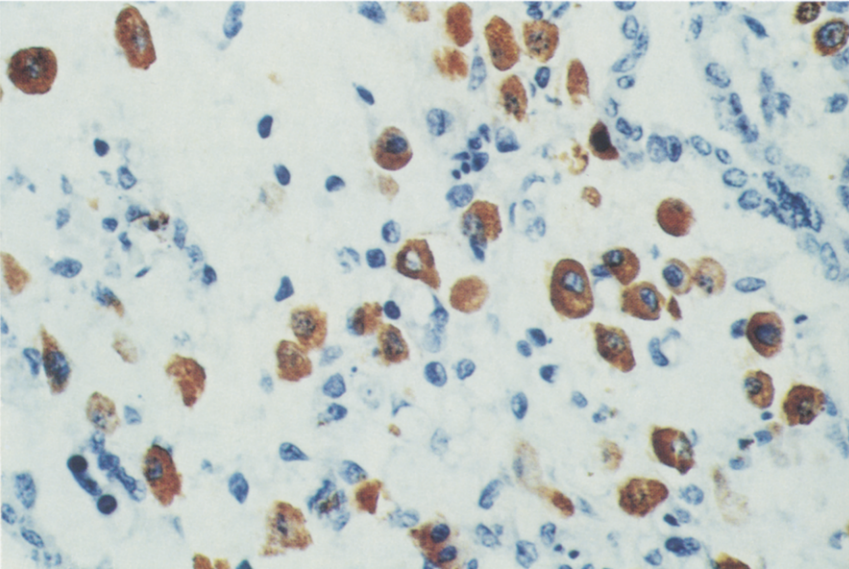
techniques are facilitated using fluorochrome-labeling due to the ease of two color discrimination by changing filters. However, immunofluroescent antibody studies of placental tissues have been infrequently used for diagnostic purposes. This may be due partly to the past limitations in using this technique in formalin-fixed tissues, and the requirement for a fluorescent microscope. The advent of inexpensive biotinylated antibodies for pathologic diagnosis has further decreased the popularity of immunofluorescent antibody methods in diagnosis. Immunofluorescence has the great advantage of rapid turnaround time, often a few hours after tissue sectioning. In our laboratories we prefer to use immunofluorescent antibody techniques to identify low numbers of microbial pathogens in formalin-fixed placental tissue. In particular, we have found this to be a highly sensitive method for tissue diagnosis of *Treponema pallidum* (Figure 3), especially when compared with the silver-based Steiner staining used in most histology laboratories (Ito et al., 1992; Hunter, 1990; Schwartz et al., 1992).

In Situ Nucleic Acid Hybridization

In situ nucleic acid hybridization adapted to tissue sections is a recently developed technique which can utilize complementary sequences of nucleic acids, either DNA or RNA, to detect and localize nucleic acids or infectious agents in tissue (O'Leary and Wright, 1991) (Table II) (Figure 4). It is a sensitive method for infectious disease diagnosis that can be rapidly performed in formalin-fixed and paraffin-embedded sections from a wide variety of tissues (Piper and Unger, 1989; Schwartz et al., 1992). It is generally used for diagnositic purposes in combination with a biotin or digoxigenin labeling technique (Leary et al., 1983; Haase, 1986; Brigati et al., 1993). In some research applications, the sensitivity of detection can be improved using a radioisotopic label such as ^{35}S or ^{32}P, with target nucleic acid detected by autoradiography (Singer et al., 1986). *In situ* hybridization is especially valuable for demonstration of infections which cannot be readily cultivated *in vitro*. Many studies have compared the relative sensitivity of *in situ* nucleic acid hybridization versus immunohistochemistry for detecting infectious agents, with varying results. However, in some cases *in situ* hybridization appears less likely to produce false negative staining due to overfixation because of the greater resistance of nucleic acids to formalin compared to antigens. Thus, *in situ* nucleic acid hybridization may be the preferred technique in placental pathology in those cases where overfixation of tissue is difficult to control. *In situ* hybridization does have some disadvantages - the reagents are more costly, the tissue digestion is more severe, and the loss of cellular and nuclear structure is more apparent, than occurs using immunohistochemistry. The *in situ* hybridization detection of RNA (such as the human immunodeficiency virus) requires prompt handling of tissue due to the lability of RNA (O'Leary and Wright, 1991).

Figure 1. Hyperplasia of Hofbauer cells can be identified in this placenta with parvovirus B19 infection by using a macrophage-specific antibody. (Immunoperoxidase with Ham56 antibody and hematoxylin counterstain, X400).

Figure 2. In this case of parvovirus B19 placental infection, double-staining immunohistochmiestry is used to identify Hofbauer cells (Ham56 antibody, red cytoplasm) and proliferating cells (Ki67 antibody, brown nuclei). Proliferating Hofbauer cells (arrows) are clearly identified within villus stroma. As expected, many proliferating cytotrophoblastic cells are present. (Immunoperoxidase with Ham56 antibody, alkaline phosphatase with Ki67, and hematoxylin counterstain, X400).
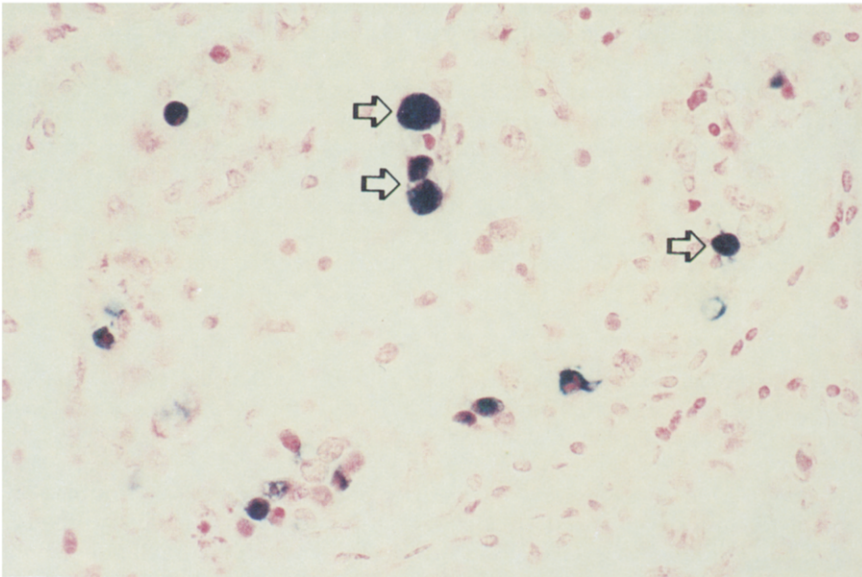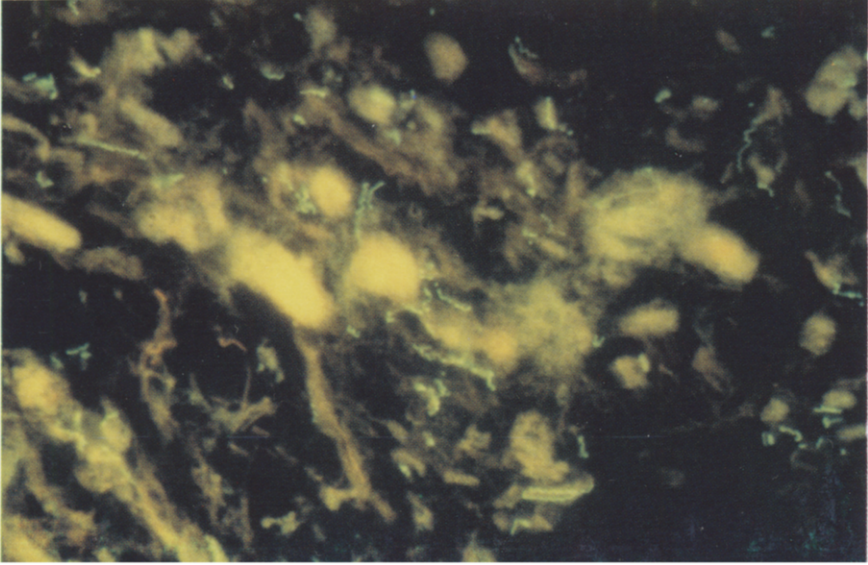
The role of *in situ* hybridization and immunohistochemistry in diagnostic placental pathology has usually been for the confirmation of infectious agents or viral cytopathic effects following examination of routinely stained sections. However, Schwartz and associates (1991, 1992) have recently used *in situ* DNA hybridization to demonstrate that herpes simplex infection of placenta-associated tissues can be found in the majority of pregnant women with concurrent or previous episodes of genital herpes. In these cases, no viral cytopathic effect can be identified in hematoxylin and eosin-stained slides, and DNA hybridization is necessary to identify the infected decidualized cells of the decidua capsularis and decidua basalis. Thus, nucleic acid hybridization can often be useful for the detection of microbial agents even in the absence of detectable organisms with routine staining.

Polymerase Chain Reaction

The polymerase chain reaction (PCR), first described in 1985 (Marx, 1988; Schochetman et al., 1988), is a technique for the *in vitro* amplification of DNA from a variety of viruses and other infectious agents. The principle of this technique involved repeated cycles of heat denaturation of target DNA, followed by annealing of primers to their complementary strands of DNA, and extension of annealed primers using a DNA polymerase. The high specificity of PCR rests on the selection of oligonucleotide sequences used to prime the reaction. Following amplification, product DNA can be detected using endonuclease restriction analysis or nucleic acid hybridization. Using PCR, a single viral genome can be detected in tissues which are both fresh as well as formalin fixed and paraffin embedded (Table III), and is thus a powerful tool with many applications for epidemiology, diagnosis, and research. In 1986, the technique was modified by utilization of the thermostabile DNA polymerase *Taq*, permitting automation of the reaction (Saiki et al., 1988). However, it still remains largely a research method which has not yet been widely used for diagnostic pathology purposes. Unfortunately, its applicability in placental pathology is limited by the inability of this technique to localize infections within tissue samples. PCR cannot distinguish maternal from fetal infections in the placenta, as both maternal (blood in intervillus space, decidua) and fetal tissues are in intimate proximity. This limitation has been primarily responsible for confusion in interpretation of early PCR-based studies of placentae from HIV-infected women (Schwartz et al., 1991). However, PCR can be used to screen for agents in placental tissues, which if positive, can subsequently be localized to specific cell types using nucleic acid hybridization or immunohistochemistry. Recently, a technique termed *in situ* PCR has been developed in which the PCR is performed directly on a slide containing a tissue section, thus combining the high sensivity of PCR with the advantages of tissue localization of *in situ* hybridization (Nuovo et al., 1991).

Figure 3. Spirochetes of *Treponema pallidum* are easily visualized in formalin-fixed tissues using this direct fluorescent antibody technique. (Monoclonal antibody to *T. pallidum* with fluorescein labeling, X1000, photograph Courtesy of Dr. Sandra Larsen, Division of Sexually Transmitted Diseases, Centers for Disease Control and Prevention, Atlanta, GA.)

Figure 4. Intranuclear inclusions of parvovirus B19 are present in this placenta stained with an *in situ* DNA probe to the virus. The infected cells (arrows) can be localized to immature erythroid cells in the blood vessels of the chorionic villus. (*In situ* DNA probe to parvovirus B19 with alkaline phosphatase, X400).

Table II

Commercially Available *In Situ* Hybridization Nucleic Acid
Probes For Infectious Agents

VIRUSES

Adenovirus
BK Virus
Cytomegalovirus
Epstein Barr Virus
Herpes Simplex
Hepatitis A
Hepatitis B
HIV-1
Human Papillomavirus (many types)
JC Virus

BACTERIA

Campylobacter jejuni
Chlamydia trachomatis
Mycoplasma pneumoniae

Table III

Commerically Available Oligo-Primers and Oligo-Probes For
Polymerase Chain Reaction of Infectious Agents

VIRUSES

BK Virus	HIV-1, 2
Cytomegalovirus	HTLV-1,2
Dengue Virus	Human Papillomavirus (many types)
Epstein Barr Virus	Japanese Encephalitis
Enterovirus	JC Virus
Hepatitis A	Respiratory Syncytial Virus
Hepatitis B	Rotavirus
Hepatitis C	Rubella
Hepatitis Delta	Varicella Zoster
Herpes Simplex	

BACTERIA

Borrelia burgdorferi	Legionella pneumophila
Campylobacter jejuni	Mycoplasma hominis
Chlamydia trachomatis	Mycobacterium tuberculosis
Escherichia coli	Treponema pallidum

PARASITES

Plasmodium falciparum	Toxoplasma gondii
Plasmodium vivax	Trypanosoma cruzi
Pneumocystis carinii	

Value of Control Samples

The importance of adequate positive and negative control samples cannot be overemphasized in the performance of all of these techniques. During the performance of the PCR technique, there are many steps when reagent or sample contamination can occur, rendering the results useless. It is thus important to intersperse negative control materials among clinical samples to identify the occurrence of contamination. When using antibody- or nucleic acid-based studies, it is important simultaneously to perform negative control slides to exclude completely the possibility of nonspecific staining. Positive control slides should consist of tissues containing the identical infection, previously confirmed by culture, serology, or other technique, which has been fixed and processed in a similar manner to the unknown clinical specimens. A control block made up of small fragments of individual tissues, each containing a different infectious agent, is often useful to exclude cross-reactivity of reagents with other microbes.

SUMMARY

A variety of highly sensitive laboratory techniques is now available for the detection and localization of infectious agents in the placenta. This communication discusses the role of immunohistochemistry, immunofluorescence, *in situ* nucleic acid hybridization, and polymerase chain reaction for infectious disease diagnosis in formalin-fixed placental tissues. These techniques, as well as other sophisticated molecular methods currently in development, will greatly facilitate characterizing the role of infectious agents in a variety of perinatal and pediatric conditions, including birth defects, intrauterine growth retardation, and stillbirth.

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