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SPECIAL TECHNICS FOR EVALUATING
FORMALIN-FIXED HUMAN PLACENTA

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KATHERINE DOROTHY WASHKO

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SPECIAL TECHNICS FOR EVALUATING FORMALIN-FIXED HUMAN PLACENTA

By

Katherine Dorothy Washko

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ABSTRACT

SPECIAL TECHNICS FOR EVALUATING FORMALIN-FIXED HUMAN PLACENTA

By

Katherine Dorothy Washko

These studies determined the value of using immunofluorescence microscopy and transmission electron microscopy for analysis of 10% formalin-fixed human placenta which is normal or affected by hemorrhagic endovasculitis.

A direct fluorescent antibody technic was used to localize immunoglobulins in normal fixed placenta. Specific fluorescence of blood vessel lumina and endothelia occurred with antiserum to IgG. Other sites of immunoglobulins were not detected despite trypsinization of tissue to restore antigenicity altered by formalin. Intense background fluorescence also obscured specific fluorescence. Determining increased deposition of immunoglobulins in abnormal tissue therefore appeared to be impractical.

Subcellular components were poorly preserved by formalin and ultrastructure was primarily used for cell identification and observations of connective tissue. Analysis of abnormal tissue was ambiguous because ultrastructural changes may have been caused by one or more of the following: autolysis, formalin, senescence, and disease.

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INTRODUCTION

A growing awareness of the effects produced by infectious agents, poor nutrition, and environmental toxins has clearly increased concern for the well-being of the unborn child and its mother. The possible correlation between these factors and unexplained reproductive failure has now stimulated more researchers to investigate not only the fetus, but the placenta as well. The placenta is a fetal organ composed of both maternal and fetal tissue and may therefore reflect the health of both the mother and her baby. It sends nourishment to the fetus from the mother and may serve as a barrier to infection and toxic substances. Placental malfunction or disease can result in serious consequences to the fetus. Numerous stillbirths and congenital disorders may be attributable to transplacental infection.

Nearly all research on the human placenta has utilized fresh tissue. The aim of this research was to evaluate the efficacy of formalin-fixed placental tissue for studies by immunofluorescence microscopy and transmission electron microscopy. A recent study of formalin-fixed placental tissue using scanning electron microscopy has shown 10% neutral formalin to be a suitable fixative for this procedure (Caetano, 1979). Hospitals throughout the State of Michigan submit abnormal placentas to the Michigan Placental Tissue Registry, Michigan State University, East Lansing, Michigan, for diagnostic and research purposes. These placentas are, for convenience, fixed in 10% neutral formalin.

It would, therefore, be important if procedures used to analyze fresh placenta could be adapted for use with formalin-fixed placenta.

Immunofluorescence microscopy has been used with normal fresh-frozen placental tissue for localizing fetal immunoglobulin and maternally-transferred immunoglobulin (McCormick et al., 1971; Faulk and Johnson, 1977; Johnson et al., 1977). The present study applied fluorescent antibody technics to formalin-fixed placental tissue in order to determine if formalin had a deleterious effect on the normal deposition and detection of immunoglobulins. Since infection stimulates a maternal and/or fetal immune response, the usefulness of this technic for evaluating infected tissue for abnormally increased amounts of antibodies could then be considered. Similarly, the feasibility of using immunofluorescence for determining an infectious cause for hemorrhagic endovasculitis (Sander, 1980) was an additional consideration.

The moderate number of descriptions of normal and abnormal placental ultrastructure have been based on fresh tissue fixed in glutaraldehyde and/or osmic acid. No reports on the use of 10% neutral formalin as a primary fixative for transmission electron microscopy were found in the literature. It would be of considerable value to be able to process placental tissue for transmission electron microscopy which had been conventionally fixed in formalin for light microscopy. Pathologic alterations can further be defined at an ultrastructural level by looking at cellular activity. These studies included transmission electron microscopy of formalin-fixed placentas in order to establish the value of 10% neutral formalin as a primary fixative for this technic. Placental tissue affected by hemorrhagic endovasculitis was also examined by this procedure.

OBJECTIVES

The objectives of these studies were:

1. To determine the effects of 10% neutral formalin on immunofluorescent technics applied to the localization of normal immunoglobulin deposition in formalin-fixed placentas.

2. To evaluate the feasibility of using immunofluorescence microscopy for determining increased deposition of immunoglobulins in infected placentas and for determining a possible infectious cause for hemorrhagic endovasculitis.

3. To determine the effects of 10% neutral formalin on placental morphology when used as a primary fixative for transmission electron microscopy.

4. To examine formalin-fixed placentas affected by hemorrhagic endovasculitis with transmission electron microscopy.

CHAPTER I

IMMUNOFLUORESCENCE MICROSCOPY

REVIEW OF LITERATURE

The Placenta - An Allograft

Paternally-derived antigens in the fetal portion of the placenta should theoretically evoke a maternal immune response during pregnancy. Failure of maternal cell-mediated immune responses to reject the placenta has stimulated researchers to investigate tolerance of this organ as an allograft. The mechanism responsible for this tolerance may be closely related to those which promote graft and tumor survival.

Urbach (1970) recognized the lack of humoral and cellular responses to placental trophoblast in postpartum women. Trophoblast cells appeared to behave in a non-antigenic manner when exposed to lymphocytes in sera obtained postpartum. He postulated that non-rejection of the placenta and fetus was made possible by a functional absence of histocompatibility antigens on the trophoblast membrane. This membrane also seemed to be an efficient barrier to the entry of sensitized maternal lymphocytes into the placenta. Youtananukorn and Matangkasombut (1972) investigated the ability of pregnant women to respond, immunologically, to placental tissue. They used the macrophage migration inhibition test to measure the response of maternal lymphocytes to pooled placental antigens. Reactivity of maternal leukocytes to placental antigens confirmed the presence of maternal cell-mediated immune responses during pregnancy, possibly stimulated by histocompatibility antigens. Sensitization of the mother could not be detected in the first trimester but

increased as gestation progressed (Youtananukorn et al., 1974). The manifestation of maternal cell-mediated immune responses indicated that an inhibitory mechanism to these responses must exist *in vivo* in order for the placenta and fetus to survive. It was theorized that antibodies mask antigenic determinants on trophoblast cells, thus suppressing recognition by maternal T-lymphocytes. A similar concept has been applied to tumor survival (Hellström and Hellström, 1970). It is thought that antibodies mask tumor antigens, thereby blocking cell-mediated immune responses. Gusdon et al. (1970) isolated a maternal antibody which reacted with impure extracts of human placental lactogen (HPL). This antibody was thought to be a blocking antibody specific for trophoblastic antigen which had not been completely separated from the HPL preparations.

It is not certain whether these masked sites on the placenta are foci of histocompatibility antigens. Bonneau et al. (1973) recovered by acid elution from the trophoblast IgG antibodies which blocked the mixed lymphocyte culture (MLC) reaction. Faulk and Jeannet (1976) also demonstrated that IgG eluates from the trophoblast inhibited MLC reactions as well as other specific and non-specific blastogenic responses of blood lymphocytes. These results indicate that blocking antibodies in the placenta are unlikely to be specific for histocompatibility antigens on the trophoblast because they inhibit other types of blastogenic responses. Such reactions may occur because lymphocytes and trophoblast cells share a similar antigen.

Stimson et al. (1979) provided further evidence in support of blocking antibodies. Leukocyte migration-inhibition assays performed on sera from abortion-prone women failed to detect the presence of a

blocking factor. This suggested that damage to the feto-placental unit in these pregnancies resulted from maternal T-lymphocyte activation.

General Distribution of Proteins in Chorionic Villi

Fluorescence microscopy of fresh-frozen placental tissue has been useful in determining the distribution of immunoglobulins and other proteins in placental villi. The possible relationship between immunoglobulin deposition and maternal immune responses against the placenta has been considered in order to explain the persistence of the placental homograft. The localization of maternal IgG has also provided insight into its mode of transfer.

Immunologic studies by McCormick et al. (1971), Faulk and Johnson (1977), Johnson and Faulk (1977), and Johnson et al. (1977) reported the general distribution of proteins in chorionic villi detected by immunofluorescent technics. Maternal IgG is the predominant immunoglobulin of placental villi. All four subclasses of human IgG are passively transferred to the fetus from the mother, but IgG_1 and IgG_3 are found in greatest quantity. Maternal IgG is faintly detectable during the first trimester because very little is transported at this time. As pregnancy progresses IgG is found extensively throughout the villous stroma, possibly as a result of the transfer process. It is present around fetal blood vessels, toward the apical aspect of blood vessel endothelia, and is also contained in the cytoplasm of mononuclear cells which populate the stroma. Loosely bound maternal IgG, eluted from the trophoblast basement membrane, was attributed with a blocking antibody function by Faulk et al. (1974) since it inhibited MLC reactions. Maternal IgG is found in fibrinoid deposits of the villous stroma in association with complement, fibrin, fibrinogen, and a small amount of

fetal IgM and IgG. Fibrinoid deposits increase during gestation and may be sites of immunologic reactivity between maternal antibodies and fetal antigens.

Fetal synthesis of immunoglobulin is minimal but fetal IgG and IgM are located in blood vessel endothelia. Fetal IgG is also distributed in mononuclear stromal cells and is tightly bound to the trophoblast basement membrane, where it may have a blocking function.

Maternal IgA is not detected within the villous stroma but is present in intervillous fibrin deposits in conjunction with maternal IgM. Complement components are also detected within stromal cells, areas of fibrinoid necrosis, and in the walls and endothelia of blood vessels. Faulk et al. (1980) reported Cl_q, C6, and C9 in blood vessel walls and C3d and C9 in the trophoblast basement membrane. The association of complement with immunoglobulin in these areas suggests activation of the maternal immune mechanism.

Numerous other proteins have been identified in the placental villus by fluorescent antibody technics. Actin, plasminogen, and transferrin are present in the trophoblast. Actin is also found in endothelial cells, stromal fibroblasts, and macrophages. Beta-2-microglobulin is located in stromal cells and endothelial cells. The most abundant protein in the villous stroma is collagen, which is distributed around placental blood vessels, throughout much of the stroma, and in basement membranes. Fibrinogen is the most commonly identified plasma protein in human placenta and is found at the periphery of blood vessels, in and beneath the trophoblast basement membrane, and in variable quantities throughout the stroma. Fibrin is located in the perivillous and intervillous space.

Placental Immunoglobulin Receptors

Placental receptors for maternal IgG have been detected on the trophoblast surface of fresh-frozen tissue by fluorescent antibody technics and by hemadsorption with antibody-sensitized erythrocytes (EA rosette formation). Matre et al. (1975) sensitized erythrocytes with human, rabbit and guinea pig IgG antibodies. This complexed IgG could be visualized bound to the surface of the syncytiotrophoblast in an agglutination pattern and suggested the presence of receptor sites for IgG on this membrane. The reaction was inhibited by primary adsorption with monomeric IgG, heat-aggregated IgG, or the Fc fragment of IgG. The inability of IgM, IgA, albumin, or other fragments of the IgG molecule to inhibit hemadsorption strongly indicates that these receptor sites are specific for IgG and that IgG binds to these sites by the Fc portion of the molecule. Van Der Meulen et al. (1980) emphasized the importance of hinge region disulfides in maintaining the binding site on IgG. Similar results obtained by Thomas et al. (1976) and Jenkinson et al. (1976) are consistent with a receptor-dependent mechanism for transport of maternal IgG and attachment of blocking antibodies for tolerance of the placenta as an allograft. The concept of Fc receptors for blocking antibodies is supported by a theory which attributes receptors on malignant cells with immune surveillance failure.

Matre and Johnson (1977) were unable to detect trophoblast receptors by immunofluorescent technics which employed conjugated IgG antibodies and theorized that widely-spaced receptors on the trophoblast could cross-link erythrocyte-bound IgG only. Wood et al. (1978a) observed that the frequency of receptors decreased in older placentas but were able to locate them by both hemadsorption and direct immunofluorescent

technics. Monomeric IgG was found to primarily bind to the trophoblast. Wood et al. (1978b) also reported attachment of erythrocyte-bound IgG to stromal cells identified as macrophages. Immunoglobulin-G Fc receptor positive macrophages only bound complexed IgG. These macrophages most likely remove immune complexes composed of maternal antibodies and fetal antigens.

The precise relationship between trophoblast membrane receptors and the transfer of humoral immunity was investigated by Balfour et al. (1978). Immunoglobulin-G labelled with ^{125}I bound to receptors on the microvillar membrane of the trophoblast and was associated with pinocytic vesicle formation. Endocytosis is thought to be the transfer mechanism for maternal IgG. Contractor (1979) discussed endocytotic processes with regard to IgG transfer but also considered intracellular digestion and phagocytosis to be important functions of endocytosis. Maternal nutrients can be transported across the trophoblast by endocytosis and phagocytosis most likely occurs as a protective mechanism for the fetus. Lin (1980) traced the passage of maternal IgG from receptor sites on the brush border to the fetal capillary during an immunoelectron microscopic study of IgG transport. Peroxidase-labelled antibodies to human IgG were used to localize IgG by the diaminobenzidine reaction. Immunoglobulin-G molecules were found to bind to the microvillar surface and to be transported across the trophoblast in endocytotic vesicles which fused with the basal membrane. Immunoglobulin-G was discharged into the interstitium by exocytosis and diffused through the capillary basement membrane. It then entered the fetal vascular lumen via endothelial gaps and interendothelial spaces.

Receptors for the Fc portion of IgG have also been detected on the endothelium of fetal blood vessels. Heat-aggregated IgG and

immune-complexed IgG bound to endothelial cells during *in vitro* studies using fluorescein conjugates (Johnson et al.,1973; Johnson et al., 1976). Receptors on blood vessels could have a protective function. They may bind maternal antibodies complexed with allotypically incompatible fetal antibodies and inhibit these antibody aggregates from entering the fetal circulation. Immune complexes composed of maternal antibodies and fetal antigens may attach to these receptors for a similar purpose. Cell membrane receptors for immune complexes also characterize cells of the immune system, such as lymphocytes and monocytes. Matre (1977) used hemadsorption to demonstrate receptor sites on fetal vessels and concluded that IgG Fc receptors on trophoblast are probably identical to those on endothelial cells. He also reported binding of monomeric IgG to these receptors, in addition to heat-aggregated and immune-complexed IgG.

Immunofluorescent Technics for Paraffin-Embedded Tissue

Unfixed frozen tissue sections present certain disadvantages for immunofluorescence microscopy. Tissues cannot be studied in retrospect, morphology is frequently distorted, and antigenic material may be lost or displaced during thawing or incubation in aqueous media. For this reason, fixed tissue seems more desirable for immunofluorescent studies. Although immunofluorescent technics have not been applied to fixed placental tissue, they have been successfully used to study other fixed tissue types.

Preservation of many antigens in fixed tissues has led to their detection with fluorescent antibody studies. Fungi were detected in formalin-fixed paraffin-embedded tissues by Miyake et al. (1968). Fischman (1969) stained rabies-infected tissue which had been fixed in

cold ethanol, methanol, or acetone and paraffin-embedded. Numerous other viruses in murine tissues resisted treatment with acetone fixation and paraffin-embedding and could be identified by immunofluorescence (Jones et al., 1971). Immunofluorescence of formalin-fixed paraffin-embedded tissue has been used to diagnose leptospirosis (Cook et al., 1971, 1972) and to identify ACTH- and STH-producing cells in human pituitary tissue (Kruseman et al., 1975). Wilmes and Hossmann (1979) also demonstrated vasogenic brain edema by immunofluorescence of serum proteins in paraffin-embedded material.

Several researchers have digested formalin-fixed paraffin-embedded tissue sections with a proteolytic enzyme prior to fluorescent staining. Kaplan and Kraft (1969) noticed enhanced fluorescent staining of pathogenic fungi in tissues which had been digested with 1% trypsin. Digestion appears to restore antigenicity previously altered with formaldehyde and to minimize non-specific background staining. Huang et al. (1976) observed that sensitivity to trypsin digestion varied with the type of tissue and background fluorescence decreased with digestion. They speculated that digestion reduced non-specific binding with fluorescein-conjugated antibodies by altering the net charge of the tissue. Digestion also seemed to expose antigenic sites in the tissue. Hall and Bagley (1978) had been unable to demonstrate *Rickettsia rickettsii* in formalin-fixed tissue until the use of trypsin digestion. Following trypsin treatment of formalin-fixed tissues, Swoveland and Johnson (1979) reported an increased intensity of fluorescent staining of viruses and Qualman and Keren (1979) detected immune complex deposition in 90% of biopsied renal tissue when compared to frozen sections. Brozman (1978) proposed that formaldehyde adequately preserved antigenic determinants and that trypsin and/or pepsin exposed antigenic sites by breaking

formaldehyde-induced intermolecular cross-links. These cross-links or methylene bridges form between amino groups of side chains of proteins and between peptide groups of the main chains, causing a conformational reorganization of proteins with subsequent loss of antigenicity. Globular proteins were not severely affected by proteolysis. Brozman suggested that digestive enzymes incompletely denature immunoglobulins in tissues by cleaving to the Fc fragment or carboxyl terminal half of the heavy chain. Immunoglobulins can therefore still be detected because antigenic determinants are present in this fragment. Eneström et al. (1980) reported that formalin preserved the antigenicity of immune deposits in renal glomeruli. Immune deposits could not be detected by fluorescent staining of non-digested tissue. However, digestion with both pepsin and trypsin not only restored antigenicity but also increased the intensity of fluorescent staining.

MATERIALS AND METHODS

Placentas fixed in 10% neutral formalin were provided by the Michigan Placental Tissue Registry. The histopathologic diagnosis accompanying each placenta influenced the selection of normal and/or abnormal placentas for each procedure. The direct fluorescent antibody technic employed was based on the procedure pf Qualman and Keren (1979) which had been applied to formalin-fixed paraffin-embedded renal tissue. Conjugated and unconjugated goat anti-human antisera were purchased from Meloy Laboratories, Inc. (Springfield, Virginia). Conjugated antisera were labelled with fluorescein isothiocyanate (FITC). A Zeiss fluorescence microscope adapted for trans-illumination and equipped with a BG 38 exciter filter and a 58 barrier filter was used for immunofluorescence microscopy.

A. Dilution of Antisera

The dilution of fluorescein conjugated anti-IgG was varied during localization of IgG in a normal placenta to determine an appropriate working dilution for FITC-labelled antisera by which specific fluorescence would be optimal and non-specific background fluorescence minimal. A working dilution for unlabelled antisera was also determined. Unlabelled antisera were used to confirm specific fluorescence. Unlabelled antibodies inhibited specific fluorescence by FITC-labelled antibodies in control slides by primary adsorption to antigenic sites. The applicability of chosen dilutions was then tested by applying diluted antisera to

tissue with a well-defined site of immunoglobulin deposition. A placenta with confirmed cytomegalic disease was used for this purpose because it contained an abundant source of immunoglobulin in numerous plasma cells.

Dilution studies were done with a placenta which had a histopathologic diagnosis of normal third trimester tissue. The second placenta characterized by abundant plasma cells had a histopathologic diagnosis of third trimester tissue with hemorrhagic endovasculitis (Sander, 1980) and cytomegalic infection.

For dilution experiments, tissue was removed from the central portion of the normal placenta between the chorionic and basal plates and dehydrated and cleared by routine histologic procedures. Tissue blocks were stored at 4°C after paraffin-embedding. Sections, 4 μm thick, were cut and briefly floated on a 43°C water bath without gelatin. They were mounted on glass slides with a non-fluorescent adhesive (SOBO glue, Slomon's Laboratories, L.I.C., New York), dried for 15 minutes at 43°C and 15 minutes at 68°C, and stored at room temperature (RT) until use.

All dilutions of antisera were made in phosphate buffered saline (PBS), pH 7.2. Each experiment tested one specific dilution of unlabelled, or adsorbing, antiserum against serial dilutions of the fluorescein-labelled antiserum. This was done to obtain a suitable combination by which specific fluorescence in test slides could be inhibited in corresponding control slides with adequate adsorption by unlabelled antibodies. Ten slides were prepared. Each of the following dilutions of FITC-labelled anti-IgG was applied to a test slide: 1:10, 1:20, 1:40, 1:80, and 1:160. Test slides had corresponding control slides. All control slides were first adsorbed with one dilution of

unlabelled anti-IgG and then exposed to the serially diluted FITC-labelled anti-IgG. Dilutions of adsorbing antiserum tested were 1:8, 1:6, 1:5, and 1:4.

Prior to immunofluorescent staining, slides were deparaffinized in two 5-minute changes of xylene, rehydrated with graded ethanol, and washed in distilled water. These solutions were chilled at 4°C. Control slides were first layered with unlabelled anti-IgG and incubated in a moist chamber at RT for 30 minutes. Sections were then rinsed in cold (4°C) PBS and washed in cold PBS for 20 minutes. Both test slides and control slides were next layered with FITC-conjugated anti-IgG and incubated for 30 minutes in a moist chamber at RT. This was followed by a rinse with cold PBS and three washes in cold PBS, 20-30 minutes per wash. Slides were partially air-dried, mounted with coverslips using glycerol-10% PBS, and examined microscopically.

Dilutions for antisera which provided optimal specific fluorescence and minimal background fluorescence were chosen to stain the placental tissue containing plasma cells. FITC-labelled conjugates were diluted 1:80 and adsorbing antisera were diluted 1:4. Tissue sections were cut from paraffin blocks which had been previously prepared for light microscopy and subsequently stored at RT. These tissue blocks were preferred to freshly made blocks since a large number of plasma cells had been observed in these tissue segments during routine histopathologic examination of the placenta. Tissue sections were cut, stained, and examined as previously described for dilution studies. However, in addition to anti-IgG, antisera to IgM and IgA were also applied to tissue.

B. Trypsin Digestion: Duration

Trypsin digestion of placental tissue was integrated into the procedure since it was theorized that proteolysis might restore antigenicity altered by formaldehyde and reduce non-specific background fluorescence. Trypsin digestion was included in the procedure of Qualman and Keren (1979). Tissue sections were trypsinized for different lengths of time beginning with 1/2 hour and 1 hour and extending to 8 hours by increments of 1 hour. Tissue was digested for 24 hours in one experiment.

Tissue samples were extracted from two placentas with a histopathologic diagnosis of normal third trimester tissue. They were processed, sectioned, deparaffinized, and stained as described in part A. However, after deparaffinization and rehydration, sections were incubated in Coplin jars at 37°C with 0.1% trypsin (bovine pancreas, type III, Sigma Chemical Company, St. Louis, Missouri) and 0.1% $CaCl_2$ in distilled water (pH adjusted to 7.8 with 0.1N NaOH). The extent of incubation varied with specific time intervals chosen for each experiment. After trypsinization, tissue sections were washed with distilled water and placed into cold PBS (4°C) overnight. Immunofluorescent staining was done the following day.

Antisera used for these experiments included anti-IgG, anti-IgM, anti-IgA, anti-fibrinogen, anti-C3, and anti-C4. A variety of antisera was employed in order to determine if exposure of antigenic sites by trypsin digestion might enable localization of immunoglobulins, complement, and fibrinogen in normal formalin-fixed placenta. Results were compared with the localization of these proteins as reported in normal fresh frozen tissue (McCormick et al., 1971; Faulk and Johnson, 1977; Johnson and Faulk, 1977; Johnson et al., 1977; Faulk et al., 1980).

Each fluorescein labelled antiserum was applied to three slides. One slide was a trypsinized test slide and the remaining two slides were controls. A trypsinized slide adsorbed with unlabelled antiserum served as one control. The second control was a non-trypsinized slide which was incubated in 0.1% $CaCl_2$. Fluorescein-labelled conjugates were diluted 1:80 or 1:40 and the unlabelled conjugates 1:4 in PBS.

C. Trypsin Digestion: Concentration Effect

The concentration of trypsin was varied to determine if the desired results unobtainable with altered digestion times (i.e., enhanced specific staining and reduced non-specific background fluorescence) could be achieved with greater concentrations of trypsin. Trypsin concentrations were varied from 0.1% to 0.7% with the same corresponding concentration of $CaCl_2$ in prepared solutions. The duration of digestion remained constant at 2 hours. Tissue sections were prepared from three placentas with a histopathologic diagnosis of normal third trimester tissue. Procedures for tissue processing, sectioning, deparaffinizing and staining were consistent with those outlined in part A. Trypsinization followed the procedure in part B.

Antiserum to IgG was applied to tissue sections because it appeared to be the only antiserum which consistently contributed to detectable fluorescence within placental villi. Fluorescein conjugates were diluted 1:80 and unlabelled conjugates were diluted 1:4. Each trypsinized test slide had two corresponding controls, one trypsinized slide which was adsorbed prior to fluorescent staining and one non-trypsinized slide incubated in $CaCl_2$ solution, the concentration of $CaCl_2$ being equal to that of trypsin.

D. Exposure Time to Fluorescein Conjugate

Considerable non-specific background fluorescence may result from adherence of unwanted fluorescein-labelled goat serum proteins to placental tissue. Exposure times to fluorescein conjugates were varied to determine if decreased exposure reduced non-specific adherence. Tissue from a normal placenta was processed, sectioned, deparaffinized, rehydrated, and subsequently digested with 0.1% trypsin for 2 hours according to the procedures described in parts A and B. Tissue sections were exposed to a 1:40 dilution of FITC-labelled anti-IgG for 30, 25, 20, 15, 10, and 5 minutes.

E. Cryostat and Freeze-Dried Procedures

A comparison between the immunofluorescence of fresh tissue and formalin-fixed tissue was made in order to assess the effects of fixation on localization of tissue immunoglobulins. A fresh placenta from an uncomplicated pregnancy was obtained from a local hospital and prepared for studies within 2-3 hours of delivery. The placenta was rinsed with distilled water and a central portion was fixed in 10% neutral buffered formalin. A second portion was cut into small segments, snap-frozen in isopentane cooled in liquid nitrogen, and subsequently stored at -70°C until cryostat sectioning and freeze-drying.

Tissue was extracted from the formalin-fixed portion, processed by routine histologic procedures, and submitted for histopathologic examination, which provided a diagnosis of normal third trimester tissue. Additional tissue was processed and sectioned for part C of these studies to establish the immunofluorescent staining pattern of this formalin-fixed placenta.

Cryostat sections, 6 μm thick, were cut at -15°C in a cryostat. Sections were mounted on slides and dried for 10 minutes for firm adhesion of the tissue to the slide. Antisera applied to tissue sections included anti-IgG, anti-IgM, anti-IgA, anti-fibrinogen, anti-C3, and anti-C4. A variety of antisera was employed so that immunofluorescent staining patterns associated with tissue proteins in unfixed placenta could be recognized and compared to those described by other investigators (McCormick et al., 1971; Faulk and Johnson,1977; Johnson and Faulk, 1977; Johnson et al., 1977; Faulk et al., 1980). The effects of formalin on the distribution of these proteins could then be considered by comparing the immunofluorescence of fixed and unfixed tissue. Each FITC-labelled antiserum was applied to two slides. One was a control slide first adsorbed with unlabelled antiserum. A 1:4 dilution was used for unlabelled antisera and a 1:40 dilution for FITC-labelled antisera. The staining procedure was consistent with that outlined in part A.

Tissues stored at -70°C were also freeze-dried with removal of frozen water by sublimation in a vacuum at -40°C. Dried blocks were directly embedded in paraffin. After sectioning at 4 μm, sections were briefly melted onto slides coated with a non-fluorescent adhesive (SOBO glue) and deparaffinized and rehydrated. Freeze-dried sections were stained in a manner identical to the cryostat sections. Freeze-drying was considered as an alternative to cryostat procedures because less displacement and/or destruction of antigens in tissue occurs with freeze-dried tissue and morphology is usually well-preserved (Gervais, 1972).

F. Layering with Goat Serum

The use of goat serum was an adjunct to many of the aforementioned experiments. Blood samples from goats were provided by Dr. A. Hall of the Department of Pathology at Michigan State University. Samples were centrifuged and serum separated. Prior to staining tissue sections with fluorescein conjugates, tissue was layered with undiluted goat serum or with a 1:4 dilution of goat serum in a manner analogous to adsorption. This procedure was an attempt to reduce non-specific tissue fluorescence by blocking non-specific adherence of fluorescein-labelled goat serum proteins with unlabelled goat serum proteins.

RESULTS

Since IgG is the predominant immunoglobulin in human placenta and its distribution has already been described in fresh tissue, working dilutions for FITC-labelled antisera were chosen based on detectable fluorescence after staining with FITC-labelled anti-IgG. This fluorescence was regarded as specific fluorescence associated with complexing of FITC-labelled anti-IgG and intrinsic IgG. Bright apple green fluorescence was primarily observed within the fetal blood vessel lumen, suggesting the presence of circulating IgG (Figure 1). Fluorescence was also observed in the blood vessel endothelium, along the surface of the trophoblast lining the maternal blood space, in the intervillous space, and in what appeared to be fibrinoid deposits on the surface of placental villi. Yellow autofluorescence of fetal erythrocytes and the trophoblast often occurred. An intense blue-green fluorescence of connective tissue was a consistent finding and appeared to be non-specific in nature (Figure 2). It clearly presented a problem for interpretation, because it could not be differentiated from specific fluorescence which may have occurred within the villous stroma and/or blood vessel walls. A working dilution of 1:80 or 1:40 for fluorescein conjugates enabled detection of some specific fluorescence and seemed to diminish non-specific fluorescence. Fluorescence of the trophoblast was no longer detected at these dilutions. While non-specific fluorescence was virtually eliminated in the placenta used for dilution studies, it

Figure 1. Normal formalin-fixed placental villi stained with a 1:80 dilution of FITC-labelled anti-IgG. Apple-green fluorescence can be seen within the blood vessel lumina (arrow). (x 313)

Figure 2. Normal placental villus. Blue-green non-specific fluorescence of the connective tissue (CT) is prominent. (x 125)

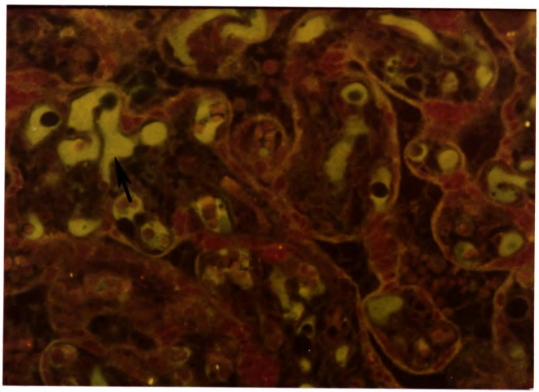

Figure 1

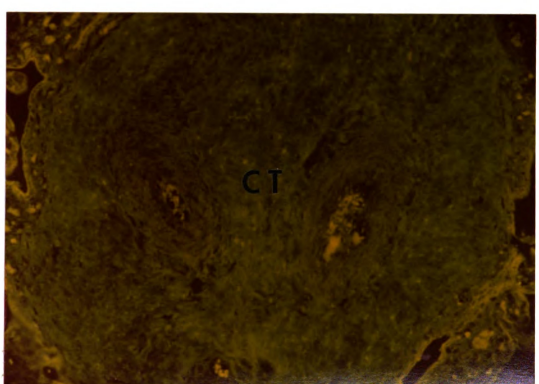

Figure 2

could not be sufficiently reduced in the majority of placentas from other experiments by dilution alone. Specific fluorescence was extremely weak at a 1:160 dilution of fluorescein conjugate and this dilution was impractical for examination and photographic purposes. Adsorption appeared to be incomplete with increased dilutions of unlabelled conjugate but was adequately achieved with a 1:4 working dilution. Fluorescence observed in test slides was inhibited in control slides and reflected primary adsorption to antigenic sites by unlabelled antibodies. However, fluorescence in control slides was not always completely inhibited due to competitive binding which occurs between labelled and unlabelled antibodies.

The strong positive fluorescence of plasma cells in the placenta with cytomegalic infection suggested that adapting immunofluorescent technics to formalin-fixed placenta might be feasible if non-specific tissue fluorescence could be eliminated (Figure 3). A 1:80 dilution of FITC-labelled anti-IgG resulted in bright fluorescence of plasma cells as well as blood vessel lumina. Globulin on plasma cells appeared to be IgG, because cells did not fluoresce with the application of FITC-labelled anti-IgM or -IgA. Only the maternal space seemed to fluoresce with these last two conjugates. Non-specific background fluorescence was observed in several tissue sections but was not apparent in adsorbed control slides. A 1:4 dilution of adsorbing antiserum sufficiently reduced fluorescence of plasma cells and blood vessel lumina in control slides, suggesting that this fluorescence was specific. Observed plasma cells may have been maternal cells which migrated through the placental barrier because fetal immune responses are primarily associated with IgM production.

Figure 3. Placental villus in tissue with cytomegalic infection. Specific fluorescence of plasma cells (arrows) occurred with a 1:80 dilution of FITC-labelled anti-IgG. Non-specific background fluorescence in this section is weak. (x 313)

Figure 4. Normal placenta digested with 0.2% trypsin. Fluorescence of the blood vessel endothelium (arrow) appears more distinct than that in non-digested tissue. (x 313)

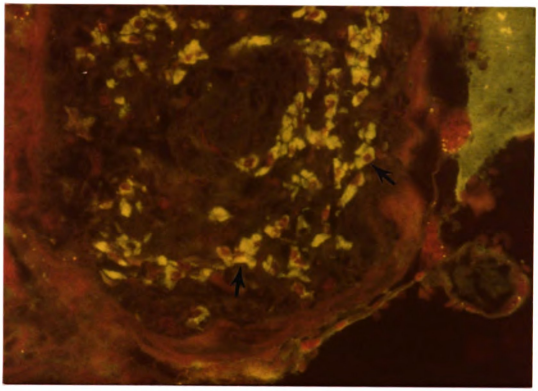

Figure 3

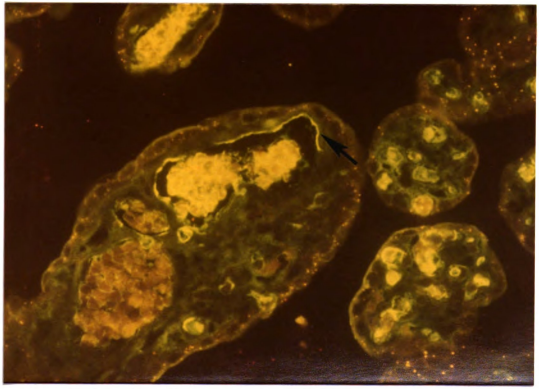

Figure 4

Increasing digestion times with a concentration of 0.1% trypsin appeared to have no effect on reducing non-specific background fluorescence. Specific fluorescence was observed in the intervillous space and blood vessel lumina with antiserum to IgG and in the intervillous space with antisera to IgM and IgA. No appreciable fluorescence was observed with antisera to fibrinogen, C3,and C4. Adsorbed control slides sufficiently inhibited the specific fluorescence observed in test slides and also had considerable non-specific background fluorescence. Tissue morphology seemed to be unaffected by extended periods of digestion at this low concentration of trypsin. The limited distribution of immunoglobulins detected and the inability to locate fibrinogen and complement suggest that increasing the duration of digestion with 0.1% trypsin does not restore antigenicity in formalin-fixed placenta. Positive fluorescence in trypsinized slides was comparable to that observed in control slides incubated in 0.1% $CaCl_2$ only.

Results obtained by increasing the concentration of trypsin during digestion were inconclusive. In general, specific fluorescence of blood vessel endothelia and lumina appeared more distinct than that in non-trypsinized control slides (Figures 4 and 5). However, additional foci within placental villi known to contain IgG did not seem to be exposed. Specific fluorescence in trypsinized test slides was adsorbed in corresponding control slides. Non-specific background fluorescence was prominent in both test and control slides but was noticeably reduced in adsorbed controls. Specific fluorescence of blood vessel endothelia and lumina appeared to be decreased with trypsin concentrations greater than or equal to 0.4% and may have reflected damage to immunoglobulin by increased proteolysis at these concentrations. Specific fluorescence was not observed with trypsinization at 0.7%. This was probably also

Figure 5. Non-trypsinized normal placenta incubated in 0.2% $CaCl_2$. Fluorescence of the blood vessel endothelium (arrow) appears to be less intense than that of trypsinized tissue. (x 313)

Figure 6. Cryostat section of normal placenta stained with FITC-labelled anti-IgG. Fluorescence of blood vessel endothelia (arrows) can be seen. (x 313)

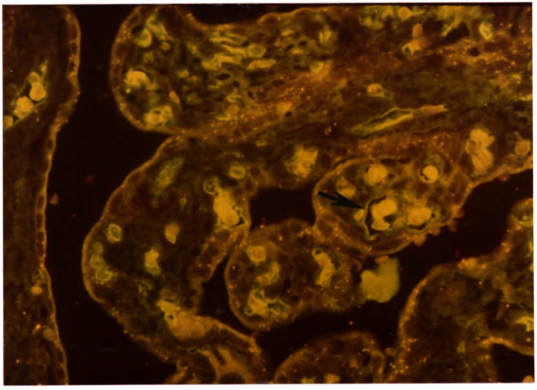

Figure 5

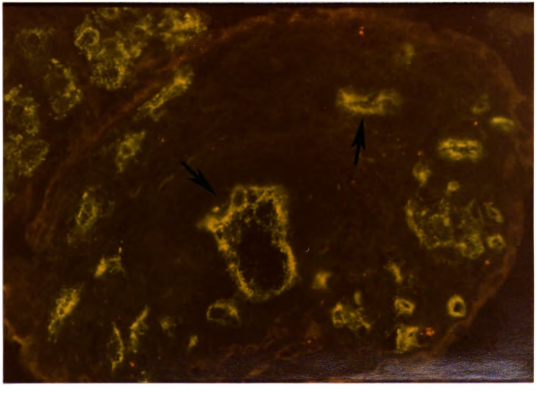

Figure 6

associated with immunoglobulin damage, because tissue morphology appeared to be disrupted at this concentration. Although specific fluorescence may have been enhanced at a concentration of 0.2% or 0.3% trypsin, evaluation of fluorescence intensity was based on subjective analysis and therefore no final conclusions were made.

Decreased exposure times to fluorescein conjugates reduced non-specific background fluorescence but also reduced the intensity of specific fluorescence. The minimal exposure time for appreciable specific fluorescence was 20 minutes. However, non-specific fluorescence was quite prominent at this exposure. Thus, shortening the period of exposure to FITC-labelled antisera was not a practical solution to elimination of non-specific background fluorescence.

Fluorescent staining patterns in cryostat and freeze-dried sections were comparable. Fluorescence, observed in blood vessel endothelia and connective tissue, appeared to be adsorbed in control slides during the application of anti-IgG antisera (Figure 6). Specific fluorescence of blood vessel endothelia with FITC-labelled anti-IgG was the only feature common to both fresh-frozen and formalin-fixed sections from the placenta obtained for comparative studies between fixed and unfixed tissue. A major difference was specific fluorescent staining of blood vessel lumina with FITC-labelled anti-IgG in fixed tissue. This was not observed in fresh tissue and was probably related to drainage of blood from vessels during cutting and freezing of the tissue. Retention of blood in the formalin-fixed portion seemed to promote strong fluorescence within the lumina, suggesting the presence of circulating IgG. Fluorescent staining of blood vessel endothelia was also observed when tissue sections were stained with antisera to IgM, IgA, fibrinogen, C3 and C4 and was adsorbed in corresponding control slides. These

results are generally inconsistent with the findings of other investigators (McCormick et al., 1971; Faulk and Johnson, 1977; Johnson and Faulk, 1977; Johnson et al., 1977; Faulk et al., 1980). Non-specific background fluorescence in cryostat or freeze-dried sections seemed to be negligible in comparison to formalin-fixed sections. Disruption of tissue morphology was more apparent with freeze-dried than cryostat sections and was probably associated with the brittleness of placental tissue which occurred with freeze-drying. Freeze-dried sections disintegrated in a water bath after sectioning. Mounting on glass slides required melting of the sections on the slides. The heat applied probably had an adverse effect on tissue morphology.

Non-specific fluorescence appeared to be diminished in the majority of adsorbed control slides and slides layered with goat serum prior to fluorescent staining. The intensity of specific fluorescence in slides initially layered with goat serum, however, was markedly diminished. Very little difference was observed between layering with diluted or undiluted goat serum.

DISCUSSION

The limited distribution of immunoglobulins, fibrinogen, and complement detected in formalin-fixed placenta with immunofluorescence probably resulted from fixation. Matre (1977) and Matre and Johnson (1977) reported an absence of receptor activity for immunoglobulins on trophoblast and endothelial cells when tissue was fixed with formaldehyde at concentrations of 4% or greater. Johnson and Faulk (1977) observed inhibited binding of fluorescein-labelled IgG aggregates to endothelia with 1% paraformaldehyde. These results indicate that receptor sites for immunoglobulin are altered in tissue fixed with formaldehyde. It is possible that other antigens in tissue, as well as receptor sites with immunoglobulin, are also altered with 10% formalin. The localization of IgG in blood vessel endothelia and lumina, and the detection of IgG, IgM, and IgA in the intervillous space, appeared to be accurate because specific fluorescence was inhibited in control slides. Results were consistent with those of other investigators who studied fresh tissue, but their findings indicated a more extensive distribution of these proteins (McCormick et al., 1971; Faulk and Johnson, 1977; Johnson and Faulk, 1977; Johnson et al., 1977; Faulk et al., 1980). They also observed IgG in trophoblast basement membranes, fibrinoid deposits, stromal mononuclear cells, perivascular areas, and throughout connective tissue. The presence of IgG within blood vessel lumina was not described and may have been attributed to drainage of

blood from fresh frozen sections. Drainage of blood from fresh frozen tissue in the present investigation was correlated with an absence of fluorescence in the blood vessel lumen. Previous investigators reported IgM in endothelia and fibrinoid deposits and the presence of IgM and IgA in intervillous fibrin deposits. Their studies located C3 in the trophoblast basement membrane, C4 in intervillous fibrin, and fibrinogen in the trophoblast basement membrane, fibrinoid deposits, and perivascular regions.

Failure to detect fibrinogen, complement components, and additional foci of IgG and IgM may have resulted from loss of antigenicity related to formalin-fixation. Brozman (1978) described preservation of antigenic determinants with formaldehyde. However, loss of antigenicity occurs due to a conformational reorganization of proteins induced by the formation of intermolecular cross-links or methylene bridges. Cross-links form between amino groups of side chains of proteins and between peptide groups of the main chains.

Trypsin was used in the present investigation to restore antigenicity previously altered by formaldehyde in order to expose additional sites of globulin deposition, fibrinogen, and complement. Brozman (1978) associated immunoglobulin detection following proteolysis with incomplete denaturation of the antibody molecule. Cleavage to the Fc fragment, or carboxyl terminal half of the heavy chain, occurs. Antigenic determinants are present in this fragment and therefore antibodies can still be detected after proteolysis.

Trypsin digestion had very little effect on formalin-fixed placental tissue. Antigenicity of immunoglobulins, fibrinogen, and complement did not appear to be restored with extended digestion nor with increased concentrations of trypsin. Specific fluorescence of blood vessel

endothelia and lumina may have been slightly intensified with trypsinization. Brozman (1978) reported increased detection of globulins in the vascular lumen with trypsin digestion. While IgG in plasma cells was readily detected in non-trypsinized placental sections, Huang (1976) and Brozman (1978) observed enhanced staining of immunoglobulins in plasma cells following proteolysis, particularly with regard to IgG. Exposure of additional antigenic sites in formalin-fixed placenta might be achieved with a second proteolytic enzyme. Pepsin has proven more efficient than trypsin in restoring antigenicity of immune deposits and enhancing fluorescence (Eneström et al., 1980). It should be considered as an adjunct to trypsin in future studies with formalin-fixed placenta because the action of trypsin alone is insufficient. Eneström et al. (1980) also reported resistance to digestion at 37°C. Placental tissue was digested at 37°C according to the procedure of Qualman and Keren (1979) and this may have contributed to the inefficiency of digestion.

Other factors may have influenced the inability to detect specific proteins. A direct fluorescent antibody technic was employed. Indirect technics are generally regarded as more sensitive and may have proven more useful in localizing proteins, like complement components, which are less easily detected. Although Wood et al. (1978b) detected C3 in the trophoblast basement membrane, Faulk et al. (1980) reported C3d in this site. C3 may not have been detected in formalin-fixed placentas because antiserum to native C3 was used. Fluorescence observed along the trophoblast at low dilutions of fluorescein conjugate was probably non-specific because it was no longer detected at working dilutions. Absence of fluorescence on the trophoblast is supported by Matre and Johnson (1977), who attributed widely-spaced IgG receptors with the

inability to cross-link fluorescein-conjugated antibodies for detectable fluorescence. Wood et al. (1978a) also detected a decrease in trophoblast receptors in older placentas.

Non-specific background fluorescence could not be eliminated. Non-specific staining of tissue with unreacted fluorescein, conjugated serum proteins, or unwanted conjugated antibodies interferes with interpretation of specific fluorescence. Serum proteins have a net negative charge which increases with conjugation by fluorescein. As a consequence, conjugated serum proteins have a strong affinity for positively charged tissue proteins. Collagen is abundant in placental villi and is probably associated with much non-specific staining. Huang et al. (1976) speculated that background fluorescence decreases after trypsin digestion because digestion alters the net charge of tissue and inhibits non-specific binding. In the present investigation, trypsin digestion appeared to have no effect on non-specific background fluorescence. Trypsin digestion combined with pepsin digestion might have eliminated this problem. Increasing the dilution of fluorescein conjugates reduced non-specific staining, but its usefulness was limited at higher dilutions. The intensity of specific fluorescence decreased at these dilutions. Extraneous proteins in unlabelled antisera and goat serum probably reduced non-specific staining by binding to tissue proteins and inhibiting non-specific binding by fluorescein-labelled serum proteins. However, initial layering with goat serum reduced the intensity of specific fluorescence. This may have resulted from adherence to specific antigenic sites by antibodies to human immunoglobulins in goat serum. Adherence of goat serum proteins to tissue in general may also have lessened fluorescence of specific antigenic sites.

For future experiments, reduction of non-specific fluorescence might be achieved by absorption of FITC-labelled antisera with acetone-dried tissue powders (Coons and Kaplan, 1950; Nairn, 1969). Proteins in dried tissue remove molecules with a strong net negative charge and therefore unwanted conjugated serum proteins and antibodies could be absorbed from labelled antisera. Conjugates should also be checked for specificity. In addition to inhibition, absorption and displacement controls should be included. With absorption, antisera are absorbed with specific antigen. Negative staining should then be achieved in this control. A displacement control involves primary layering with FITC-labelled antiserum followed by layering with unlabelled antiserum. If conjugated antibodies are specific, competitive binding between labelled and unlabelled antibodies causes displacement of labelled antibodies with diminished fluorescence. Immunoelectrophoresis could also be used to determine specificity of conjugates.

Fluorescence of the villous stroma and blood vessel endothelia in cryostat and freeze-dried sections with FITC-labelled anti-IgG was consistent with known IgG deposition in fresh-frozen placental villi. However, strong fluorescent staining of blood vessel endothelia was also observed with FITC-labelled anti-IgA, anti-IgM, anti-fibrinogen, anti-C3, and anti-C4. Endothelia were the only fluorescent sites associated with these antisera. This suggests that fluorescence in these sections may have resulted from non-specific binding of conjugated antisera to endothelia. Immunoglobulin-A, fibrinogen, C3, and C4 have not been observed in blood vessel endothelia by previous investigators. Immunoglobulin-A and C4 have been detected in the intervillous space, fibrinogen in stromal and perivascular areas, and C3 in trophoblast basement membranes. Immunoglobulin-M has been observed in the endothelium.

The possibility exists that fluorescence of endothelia with FITC-labelled anti-IgG and anti-IgM was specific, but this is indeterminable due to similar patterns of fluorescence observed with the other antisera. Non-specific adsorption of FITC-labelled anti-IgG could have been determined by washing sections at 37°C for 4 hours to remove IgG attached to endothelial receptors (Wood et al., 1978a). Fluorescence with labelled anti-IgG would have been subsequently absent if staining was specific.

Non-specific fluorescence of the endothelia may have been caused by immunoglobulin aggregates in the fluorescein conjugates. Aggregates may form upon storage and non-specifically attach to tissue sites during staining. Future experiments employing antisera should routinely include filtering of antisera with a sterile 0.45 μm filter to remove aggregates (Johnson et al., 1976). Antisera should also be ultracentrifuged at 143,000 x g for 90 minutes and used at a final dilution of 1:100 or greater (Johnson et al., 1976; Johnson and Faulk, 1977). Dilutions of antisera should contain less than 0.5 mg/protein/ml (Wood et al., 1978b). In addition, sections from fresh tissue should be adequately washed in PBS prior to staining to eliminate blood elements which non-specifically bind antisera. Adsorption of control slides suggests that aggregates may have also formed in unlabelled antisera. Thus, inhibition of fluorescence could have occurred with primary binding by unlabelled aggregates.

It appears that immunofluorescence microscopy of formalin-fixed placenta has limited usefulness in estimating increased immunoglobulin deposition associated with disease. Formalin-fixation and non-specific tissue fluorescence complicate detection and interpretation. If non-specific tissue fluorescence can be eliminated, the application of

anti-viral antisera might be possible in cases of suspected viral infection. Detection of antigens associated with cells was readily apparent with fluorescent staining of plasma cells. Localization of intracellular viruses is feasible if the intensity of surrounding non-specific fluorescence is reduced sufficiently to enable detection of specific fluorescence within cells. The use of epi-illumination for fluorescence microscopy should also be considered. Incident light might lessen the effect of non-specific fluorescence.

With regard to hemorrhagic endovasculitis, detection of increased deposition of immunoglobulins in areas of vascular damage appears to be impractical with formalin-fixed placenta. Non-specific fluorescence cannot be differentiated from specific fluorescence and formalin masks antibody sites. However, elution studies could be performed in order to quantitate IgG and IgM in affected placentas (personal communication, Pienkowski, 1981). Antibodies should be eluted from both unfixed normal and abnormal placental homogenates and then quantitated. The relative quantity of immunoglobulins characterizing normal tissue could then be estimated and compared with that of the abnormal tissue to determine if levels are increased with disease. Accessibility to mothers should be possible in order to identify immunoglobulins as fetal or maternal by allotypic determinations. Immunofluorescence microscopy could then be applied to normal and abnormal tissue and antibody concentrations correlated with the degree of specific fluorescence.

It is important to note that immunofluorescent technics were applied to formalin-fixed tissue with the intent of localizing immune complexes in damaged blood vessel walls of placentas affected by hemorrhagic endovasculitis. It was speculated that soluble immune complexes might be the cause of vascular damage. However, it should be stressed

that in cases of twinning or other multiple births, hemorrhagic endovasculitis is often associated with only one portion of the placenta and the fetus subtended by it. This strongly suggests that immune complex disease is not the cause of hemorrhagic endovasculitis since immune complexes should affect the entire placenta and all fetuses (personal communication, Pienkowski, 1981). It is interesting to speculate on the relationship between hemorrhagic endovasculitis and the site of placental attachment and fetal development *in utero*. With multiple births, the portion of the placenta with vascular damage and the fetus subtended by it may be subject to injury due to a less favorable site in the uterus (personal communication, Pienkowski, 1981). It also seems possible that this might often occur with single births.

In conclusion, immunofluorescent technics appear to be impractical for determining increased deposition of immunoglobulins in placentas with hemorrhagic endovasculitis or other abnormalities, particularly when formalin-fixed tissue is used. Quantitative studies of fresh tissue should be correlated with fluorescent studies. Furthermore, immunofluorescence microscopy cannot be applied to formalin-fixed placentas unless the effects of non-specific fluorescence and formaldehyde are eliminated.

CHAPTER II

TRANSMISSION ELECTRON MICROSCOPY

REVIEW OF LITERATURE

Placental Villous Architecture

The functioning unit of the placenta is the placental villus. A villus in cross section reveals the circular profile of a rather loosely textured connective tissue core composed of connective tissue cells, collagen fibers, and blood vessels. The stromal core is bounded by an epithelial covering, the trophoblast membrane. This epithelial membrane is composed of an inner layer of cytotrophoblast cells and an outer layer of syncytial trophoblast cells. Maternal blood, found external to the epithelium in the intervillous space, bathes the placental villus. It is across this membrane that fetal waste products are exchanged for maternal oxygen and nutrients.

Kaufmann et al. (1979) provided an excellent summary of the branching architecture of the placental parenchyma. The peripheral villous tree is composed of stem villi, intermediate villi, and terminal villi. Stem villi have a fibrous stroma containing arteries, arterioles, capillaries, venules, and veins. Stem villi are highly branching structures and give rise to intermediate villi. Intermediate villi branch into terminal villi and are thought to provide the terminal supporting system. Unlike vessels of stem villi, blood vessels of intermediate villi lack a tunica media and are supported by a looser form of connective tissue. Terminal villi comprise the final branchings of the villous tree. A few reticulum cells and fibers are present in the stroma, which is mainly

composed of large sinusoidal capillaries. These capillaries bulge into the epithelium causing it to become distended. This forms a very thin barrier, or "epithelial plate", between the fetal capillary and maternal blood space and constitutes an efficient exchange site.

Normal Placental Ultrastructure

Boyd and Hughes (1954) made early basic observations of human placental ultrastructure. A more detailed description of the early and mature placental labyrinth (branching villi) was given by Wislocki and Dempsey (1955). It included the peripheral cytotrophoblast of the anchoring villi, which attaches to the decidua. Cells in this area differ morphologically from those of the labyrinth.

Immature placentas were described by Sawasaki et al. (1957), Terzakis (1963), Lister (1964), Dec and Bartel (1976), and Demir (1980). The placenta is highly active in absorptive and resorptive processes and a well-developed brush border lines the free surface of the syncytium bordering the maternal blood space. The brush border is a significant morphologic feature as it performs transport functions similar to those in the epithelium of the intestine and renal tubules. The brush border is composed of abundant long microvilli which are rod-like in shape with swollen, round, or oval tips. Microvilli also cover cytoplasmic promontories which protrude into the intervillous space. They greatly increase the absorbing and secretory surface of the syncytium. Microvilli also project into the intercellular space between syncytial and cytotrophoblast cells.

The syncytial cell is more electron dense than the cytotrophoblast cell. The syncytial cytoplasm is extremely vacuolated and contains a substantial number of electron dense granules. The marked vacuolization

increases during the course of pregnancy and appears to be associated with resorption, absorption, and hormonal function. Two main types of vesicles or vacuoles are found. The larger of the two is associated with microvilli and appears to form during pinocytosis. The pinocytotic vesicles have an irregular membrane and often contain a flocculent material. The second type of vacuole is more uniform in size with a well-defined beaded or granular membrane. This is a vesicular form of endoplasmic reticulum with ribonucleoprotein particles on the membrane. Elongated cisternae may also be present as well as smooth endoplasmic reticulum. Numerous free ribosomes are found in the cytoplasm.

The most frequently mentioned electron dense granule in the syncytial cytoplasm may be a nutritive lipid. It is present at all levels extending from the trophoblast and its basement membrane to the fetal capillaries and connective tissue. This granule clearly has no distinct membrane or internal structure. Most authors believe that syncytial cells manufacture steroid hormones. Terzakis (1963) considered the relationship between granules and hormone synthesis. He described moderately electron dense granules with a distinct limiting membrane originating within the Golgi apparatus. These may be secretory granules containing estrogen or progesterone and are quite numerous at term. Terzakis also described lysosome-like granules and a fourth type of granule containing both low electron dense material and electron dense lamellae.

Other features of syncytial cells during early pregnancy are Golgi zones, intercellular membranes, numerous small oval mitochondria with well-defined cristae, and glycogen granules. Cell nuclei assume irregular shapes and the nuclear membrane is lined with granular

chromatin. Fine filaments of fibrous protein are present in the center of the cell and at the apical and basal surfaces. Vacek (1969) compared them to the tonofilaments of other epithelial cells which serve a cytoskeletal function. They also converge into desmosomes and possibly strengthen cell attachments. Vacek reported an increase in syncytial tonofilaments in pregnancies where contraception by intra-uterine devices failed and thought they might represent cellular degeneration or be related to metabolic activity.

Cytotrophoblast cells (Langhans cells) form a continuous layer in early pregnancy. Intercellular clefts between these cells and syncytial cells suggest areas of fusion, supporting the concept that Langhans cells give rise to syncytial cells (Boyd and Hamilton, 1966; Metz et al., 1979). The Langhans cell is less electron dense and less vacuolated than the syncytial cell. It has larger mitochondria with an electron dense matrix and many Golgi zones. The small amount of endoplasmic reticulum assumes an elongated form. Abundant ribosomes and polyribosomes are found free in the cytoplasm along with particulate glycogen and lipoid droplets. Terzakis (1963) described secretory-type granules in the cytoplasm and human chorionic gonadotrophin may be synthesized by these cells (Dec and Bartel, 1976). Round or elliptical nuclei have agranular chromatin and prominent nucleoli. Tonofilaments are present in the cytoplasm and desmosomes attach adjacent cells.

Distinct morphologic differences which occur in the placenta during the latter part of pregnancy reflect changes in functional demands and metabolic activity. Numerous investigators have reported observations on the aging and mature placenta (Wislocki and Dempsey, 1955; Sawasaki et al., 1957; Rhodin and Terzakis, 1962; Zacks and Blazar, 1963; Boyd and Hamilton, 1970; Dec and Bartel, 1976; Demir, 1980).

Syncytial cells show a remarkable variation. The surface lining the intervillous space becomes smoother with shorter cytoplasmic promontories and fewer microvilli. The small microvilli terminate in enlarged or bulbous tips. The number of pinocytotic vesicles is reduced. Fusion of cell membranes produces a true syncytium. Syncytial nuclei have nucleoli and dense granular chromatin. Aggregates of nuclei may form which decrease the width of adjacent portions of cytoplasm. Jones and Fox (1977) described these "syncytial knots" and attributed them to degenerative processes resulting from hypoxia or ischemia. They also described their association with the formation of intervillous bridges. Metz, Weihe, and Heinrich (1979) discussed the intercellular junctions attaching intervillous bridges. Further dilatations of endoplasmic reticulum result in numerous cisternae. They contain material of low electron density and enhance the vacuolated appearance of the syncytial cytoplasm. Ashley (1965) noted the association of endoplasmic reticulum cisternae with membranes of an intracellular canal system. He described membrane-bound narrow canals extending through the syncytial cytoplasm by which substances can be transported in a highly selective manner following pinocytosis. Particles not absorbed from the canals by the cytoplasm travel across the syncytium and pass through the intricate basal folds of the cell membrane to the fetal circulation below. Fewer ribosomes, mitochondria, lipoid droplets, and Golgi complexes are found. Myelin bodies and autophagic vacuoles are degenerative features. Secretory granules are present. Masses of fibrin adhere to the external plasma membrane in aging tissue.

The number of cytotrophoblast cells is greatly reduced in term placentas and they no longer form a complete layer. There is a scarcity of endoplasmic reticulum, Golgi zones, free ribosomes, and mitochondria.

A minimal amount of granular chromatin accumulates beneath the nuclear membrane. Infoldings of the plasma membrane, as in the syncytial cell, increase the surface area for efficient fluid transport through the epithelium.

Vasculo-syncytial membranes are thin barriers which separate the fetal and maternal blood. They form in late pregnancy when syncytial nuclei aggregate, thereby creating thin areas of cytoplasm which overlay fetal capillaries and are free of nuclei. This facilitates fetal-maternal exchange as fetal blood vessels are in close proximity to the maternal space. Pisarski and Topilko (1966) included the following five layers in this barrier: a thin layer of syncytiotrophoblast cytoplasm without nuclei, the trophoblast basement membrane, a fine layer of connective tissue containing collagen, the capillary basement membrane, and the endothelial cell cytoplasm.

Verbeek, Robertson, and Haust (1967) observed basement membranes in term placentas. Martinek, Gallagher, and Essig (1975) described those of fetal capillaries. The epithelial basement membrane is composed of a fine fibrillar material which tends to thicken as the placenta ages. It consists of a lamina lucida and a lamina densa and is possibly a secretory product of the trophoblast cells. The capillary basement membrane is highly irregular and ill-defined. It consists of a lamina lucida and a fragmented lamina densa. It also increases in thickness with placental senescence. It may have a single layer or multiple individual layers which branch and anastomose. Collagen fibrils are present in between the component layers. The fetal capillary basement membrane is often thicker than that of the trophoblast epithelium. These two types of basement membranes can be seen to fuse in vasculo-syncytial membranes.

The placenta is a highly metabolic organ. It has absorptive and secretory functions and synthesizes proteins, carbohydrates, fatty acids, and nucleic acids. It is a source of human chorionic gonadotrophin, progesterone, and estrogen. Studies on specific structures and specialized regions in the syncytiotrophoblast suggest that the trophoblast is responsible for these processes. Burgos and Rodríguez (1966) described an alpha zone and a beta zone in the trophoblast. The alpha zone is a thin zone for transport in both directions. Microvilli on its syncytial surface are associated with vesicles, pinosomes, caveolae, and canaliculi at their base. Basal infoldings in this zone also indicate fluid transport. Ockleford and Whyte (1977) isolated coated vesicles which followed the distribution of microvilli and were implicated in protein absorption. A major protein extracted from their surface, possibly clathrin, may prevent their fusion with lysosomes. Ockleford and Menon (1977) also investigated attachment sites on the syncytial surface for iron. An absence of vesicular uptake indicated that iron bound to receptors in specific regions and was internalized through the membrane.

The beta zone is a thicker zone. The organelles related to cellular synthesis are well-developed in this region. The dilated cisternae of endoplasmic reticulum contain material of low electron density and are associated with canaliculi and dense granules. Yoshida (1964) thought that the electron density of these secretion granules resulted from their protein nature. He also described their relationship to the endoplasmic reticulum and the Golgi apparatus which may synthesize them. He speculated that they liquefy at the syncytial surface and release their contents into the maternal blood. These granules might represent steroid hormone production.

Morphologic differences between the fetal and maternal surface of the mature placenta were reported by Lister (1963a,b). Features of placental aging are more prominent on the maternal surface. Microvilli are fewer and smaller. The number of granules, organelles, and vesicles in the cytoplasm of trophoblast cells is markedly reduced, suggesting diminished transport function and hormone synthesis in late pregnancy. The syncytium is thin, the trophoblast membrane thickens, and stromal collagen fibers are numerous. In contrast, the fetal surface resembles the early placenta because its features indicate metabolic activity and absorption. The syncytial surface has well-developed microvilli and many pinocytotic vesicles. Cellular organelles and other cytoplasmic components are abundant. Unlike the maternal surface, syncytial nuclei are well-spaced and do not aggregate. The number of Langhans cells is greater.

The fetal capillary endothelium is important during the exchange process for it is the final monitor of substances passing to the fetal circulation. Caveolae and micropinocytotic vesicles occur at both the luminal and stromal surfaces. They appear to be associated with the transport of materials to and from the capillary lumen across the cytoplasm. Endothelial cells are eccentrically located in the villus. They have cytoplasmic extensions in close proximity to the trophoblast basement membrane which may facilitate transfer. The cytoplasm contains mitochondria, granular endoplasmic reticulum, Golgi membranes, pinocytotic vesicles, glycogen, polysomes, and fine filaments. Pericytes, encircling the endothelial cells, resemble them but have dilated sacs of granular endoplasmic reticulum. Endothelial cells are usually attenuated but may thicken during constriction associated with circulatory regulation. The association of the fine filaments or microfibrils

with contractile or cytoskeletal functions has been investigated. Filaments were initially reported by Zacks and Blazar (1963). Terzakis (1963) observed that they were less distinct in constricted capillaries than in relaxed vessels. He proposed that shortening of these parallel filaments caused constriction. Vacek (1969) noted the abundance of tonofilaments in the cytoplasm of endothelial cells and trophoblast cells and considered a possible cytoskeletal function for them.

Human umbilical vessel endothelium has microfibrils similar to those of villous capillary endothelium. Parry and Abramovich (1972) and Asmussen and Kjeldsen (1975) reported the similarity of microfibrils in the endothelium of umbilical arteries to the myofilaments of the underlying smooth muscle. Studies of endothelial cell cultures derived from umbilical veins were done by Jaffe et al. (1973) and Elgjo et al. (1975). Cells contained actomyosin of the smooth muscle type, suggesting a contractile function for microfibrils.

In addition to microfibrils, Heinrich et al. (1976) considered the association of intercellular junctions with contractility in the fetal capillary. He reported tight, gap, and adherent junctions and compared the continuous capillary endothelium of human placenta with that of muscle and brain. Adjoining endothelial cells overlap extensively, resulting in interdigitating cytoplasmic processes. It is possible that endothelial constriction is controlled by the pseudopodial extensions between overlapping cells. Overlapping produces gap junctions with intercellular clefts. Tight junctions are found near the lumen. Metz and Weihe (1980) also associated gap junctions with vascular dynamics. They thought that both endothelial and myoendothelial cells altered the diameter of the vascular lumen. Gap junctions found between adjacent endothelial cells, endothelial and smooth muscle cells, and adjacent

smooth muscle cells could contribute to coordinated contraction.

Microfibrils are characteristic of blood vessel endothelium in many types of tissue. Their precise function remains unresolved. Rhodin (1962) described the noticeable change in fine structure of vascular endothelium in mammals during transition from arteriole to capillary. The capillary no longer has a muscular media and its contractile ability is related to new functional properties of the endothelium. He correlated microfibrils with myofilaments. Both Rhodin and Cecio (1967) also speculated on the supportive function of these filaments in mammalian endothelial cells. They might confer elasticity to cells subject to pressure. Hammersen (1980) reviewed concepts on endothelial contractility and favored a cytoskeletal function of microfibrils as they are increased in endothelial cells which must sustain considerable degrees of mechanical stress.

The architecture of the villous stroma consists of connective tissue cells, fibers, and fetal blood vessels. Kaufmann, Stark, and Stegner (1977) provided a detailed description of fixed connective tissue cells in the placenta. Highly proliferative mesenchymal cells are present in the stroma during the first trimester. They differentiate into reticulum cells which predominate in terminal villi and fibroblasts which predominate in stem villi. However, reticulum cells form the majority of stromal cells during the first two-thirds of gestation. At this time, a meshwork of reticular and collagen fibers encompasses the fetal blood vessels. Stromal channels constitute the remaining interspaces and are fluid-filled compartments which contain the placental macrophages or Hofbauer cells. Hofbauer cells are extremely vacuolated and have funnel-like cytoplasmic extensions which suggest their macrophagic nature (Castellucci et al., 1980). In the last trimester of pregnancy

stromal channels in the stem villi are replaced by masses of fiber. In the terminal villi they are replaced by sinusoidal enlargements of fetal capillaries.

Mesenchymal cells in the young placenta are small spindle-shaped cells attached to each other by long cytoplasmic processes. They have very little cytoplasm and few organelles.

The most numerous connective tissue cell in mature placental villi is the small reticulum cell. Its contour is highly irregular and it has long branching cytoplasmic processes. The processes of different cells anastomose and divide the stroma into chambers. This cell is characterized by richly developed vesicular endoplasmic reticulum and has little cytoplasm. Large reticulum cells have few cytoplasmic extensions but abundant cytoplasm.

Small reticulum cells are the precursors of fibroblasts, the connective tissue cells of third trimester stem villi. During transition to the fibroblast form, cytoplasmic processes become fewer and shorter and the cisternae of endoplasmic reticulum become confluent.

Fibroblasts produce collagen fibers which are abundant in stem villi. Unlike small reticulum cells, fibroblasts have a large amount of cytoplasm and just a few thick short processes. The elongated fibroblast is filled with a confluent network of well-developed endoplasmic reticulum and numerous ribosomes cover its dilated cisternae. Intracellular filaments, mitochondria, and glycogen are the most frequently seen cytoplasmic constituents.

The development of the connective tissue fibers was described by Kaufmann et al. (1977) and Münch (1978). The reticular villous stroma of early pregnancy has a dense meshwork of reticular fibers entwined with collagen fibers. These fibers enter basement membranes and

accumulate beneath them. During maturation early fibers are replaced by bundles of thick collagen fibers. Collagen fibers are numerous in stem villi, where they provide strong mechanical support. They are also found external to fibroblasts. The sinusoidal type of stroma of terminal villi is primarily composed of dilated capillaries supported by a minimal amount of reticulum cells and tissue fibers. This type of stroma facilitates exchange between the maternal and fetal circulations.

Smooth muscle cells were reported in human placentas by King and Gröschel-Stewart (1965) and Babcock (1969). Muscle fibers are randomly distributed in the stroma or attached to blood vessels. They may contact adjacent cells by gap junctions (Metz and Weihe, 1980). The extraction of a contractile protein closely related to skeletal actomyosin suggests that these cells influence placental circulation.

The human placenta is hemochorial. The fetal chorion invades the uterine tissue to attach the placenta mechanically and to establish contact with the uterine blood vessels. The morphology of the anchoring villi in this junctional zone was described by Enders (1968), Moe (1969), and Dempsey, Lessey, and Luse (1970). The anchoring villus in the decidua has cytotrophoblastic cell columns which are distal to the stromal core. The cytotrophoblastic shell is a layer of cells which form when the cell columns contact the decidua. Cytotrophoblast cells may phagocytize or erode the decidua. Isolated masses of syncytium attached to the cytotrophoblast cells have long straight microvilli and pinocytotic vesicles. They appear to engulf the detritus of the decidual cells. These cytotrophoblast cells differ morphologically from those which are adjacent to the villous stroma. They have an abundance of glycogen, filaments, and pronounced endoplasmic reticulum. A granular to hyaline intercellular material separates the cells and is

designated as fibrinoid. It is probably composed of fibrin, cellular debris, maternal plasma proteins, and a secretory product of the cytotrophoblast cells.

Placental Ultrastructure Associated with Complications of Pregnancy

Normal placental senescence results in decreased placental activity. Pregnancies which extend to or beyond 42 weeks are considered prolonged. The risk of fetal growth failure, hypoxia, malnutrition, and perinatal death increases as a result of diminished transport functions in the placenta. Jones and Fox (1978a) described the trophoblast in prolonged prengnancy and attributed unusual changes to decreased uteroplacental blood flow which occurs after the fortieth week of gestation. They observed degenerative changes in the syncytium which are features of normal placental aging but also reported necrosis with adjacent areas of proliferating microvilli. Trophoblastic alkaline phosphatase, associated with transport mechanisms, was elevated. Cytotrophoblastic hyperplasia occurred and an increased number of cytoplasmic organelles indicated cellular activity. These changes may be a placental response to ischemia. Capillary endothelial cells were abnormal. They were highly contracted with an overabundance of organelles and surface blebs. The villous stroma contained a large amount of fibrillary material, collagen, and numerous vesicles. Thliveris and Baskett (1978) reported increased syncytial necrosis and fibrinoid deposition in prolonged pregnancy and a reduction in enzyme activity in necrotic areas.

Morphologic changes occur in placentas from hypertensive pregnant women. Thliveris and Speroff (1977) observed cytotrophoblastic hyperplasia with increased syncytial degeneration and fibrinoid deposition. Large amounts of collagen were found in fibrinoid areas and the trophoblast

basement membrane was thickened. These changes were attributed to hypoxia resulting from occlusion of maternal spiral arteries by fibrinoid material and lipid-laden cells. Dec et al. (1977) reported similar findings. Their study also described numerous myelin structures in the syncytium and damage to the endothelial cells of blood vessels. Intraluminal thrombi were found and the endothelial cells contained an abundance of microfilaments.

Zacks and Blazar (1963) found no significant changes in placentas from mothers with diabetes mellitus. Lister (1965) studied placentas from diabetic mothers which were associated with abnormal fetal outcome. Numerous Langhans cells were present. The clear appearance of many of them suggested cell death. Swollen mitochondria were present in the syncytium and capillary endothelium. Capillary endothelial cells contained large groups of filaments and crystalline inclusions. Fibrinoid degeneration and possible thickening of basement membranes were also described. Okudaira et al. (1966) reported an inconsistency in their own findings but additionally noted an increased cellularity of the stroma which contained deposits of a basement membrane-like material and large vesicles. Hemorrhage into the villous stroma was observed in one placenta with diapedesis of a red blood cell through the capillary wall. Similar observations of placentas associated with diabetes were reported by Widmaier (1970).

Pre-eclampsia of pregnancy may cause growth failure or intrauterine fetal death. Zacks and Blazar (1963) failed to recognize any morphologic abnormalities in placentas related to pre-eclampsia. Observations by Jones and Fox (1980) indicated significant changes which were correlated with decreased uteroplacental blood flow. The syncytium showed necrosis, few secretory granules, swollen mitochondria, and a

quantity of dilated rough endoplasmic reticulum which surpassed that of normal placentas. Proliferating cytotrophoblast cells were replenishing the syncytium as indicated by intermediate transitional forms. Occasional degenerating cells were present. Trophoblast basement membranes were markedly thickened. Small capillaries had swollen endothelial cells characterized by an increase in organelles and filaments. Vesicles and collagen were abundant in the stroma.

Morphology of the placenta in erythroblastosis was investigated by Widmaier (1969). He reported wide syncytial bridges between villi, destruction of the syncytiotrophoblast, numerous Langhans cells and Hofbauer cells, and thickened basement membranes. Jones and Fox (1978b) considered the trophoblast as a site of immunologically mediated damage but failed to see evidence of immune complexes. They described degenerated syncytial nuclei as myelin bodies and reported cellular hyperplasia and abundant collagen in an edematous stroma. Thick immature endothelial cells had numerous organelles, cytoplasmic blebs, and extremely thickened basement membranes.

Placental ultrastructure associated with poorly defined complications has also been documented. The continued viability of the trophoblast after fetal death was studied by Davies and Glasser (1967). They described a functioning trophoblast and attributed its survival to accessibility to maternal blood. The villous stroma of these placentas became fibrosed, containing collagen and basement membrane-like material. The endothelium of some fetal vessels remained intact with few organelles but other vessels became sclerosed. Complete fibrous obliteration of stem vessels during placental persistence has also been reported with light microscopy (Theuring, 1968). Kemnitz and Theuring (1974) described some features of placentas associated with retardation of birth and

concluded that retardation could not be diagnosed purely by morphologic analysis.

MATERIALS AND METHODS

Nine placental specimens fixed in 10% neutral formalin were provided by the Michigan Placental Tissue Registry. Specimens had been maintained in 10% neutral buffered formalin for three to nine months prior to processing for transmission electron microscopy. The histopathologic report which accompanied each placental case provided evaluation of the tissue as determined by light microscopy.

Five placentas were from liveborn infants near or at term. Villous histology was consistent with normal third trimester placenta. No specific tissue alterations involving inflammatory or degenerative processes were observed.

Four placentas were from stillborn infants near or at term. Each had a histopathologic diagnosis of third trimester tissue severely affected by hemorrhagic endovasculitis and hemorrhagic villitis (Sander, 1980). Small terminal villous vessels were particularly affected in one placenta and large stem vessel involvement occurred in the other placentas. Chronic villitis of unknown etiology, as described by Russell (1980), also accompanied the vascular lesion in one placenta. Some pathologic alterations of blood vessels reported by light microscopy were non-exudative necrosis of vessel walls, fragmented red blood cells with diapedesis and hemorrhage into the villous stroma, thrombosis, intimal proliferation, and fibrosis. Other abnormal villous features included stromal hypercellularity and edema, areas of infarction and

ischemic necrosis, villous fibrosis, intervillous thrombosis, erythroblastosis, and intranuclear inclusions associated with cytotrophoblast cells and connective tissue cells.

Pieces of tissue, 4 mm^3 in size, were removed from randomly chosen sites in the central portion of the placenta between the chorionic and basal plates. Samples were initially washed for 10 minutes in Zetterqvist's wash solution (Pease, 1964) to remove residual formalin. The wash solution consisted of veronal acetate buffer and a balanced salt solution. The pH was adjusted to 7.2-7.4 with 0.1N HCl. The tissue was then post-fixed for two hours in Zetterqvist's osmium fixative (Pease, 1964). The fixative was prepared by dissolving 1 gram of osmium tetroxide in approximately 98 ml of Zetterqvist's wash solution. A second 10 minute buffered wash was followed by dehydration. Samples were passed through a series of 50%, 75%, 95% and 100% ethanol. One hundred percent ethanol was gradually replaced by 100% propylene oxide during stepwise increments of the propylene oxide to alcohol ratio (1:2,1:1, 2:1, and 3:0). This was followed by infiltration of the tissue with a resin mixture of Epon 812-Araldite in a similar stepwise substitution sequence in order to replace the propylene oxide with resin. Tissue samples were reduced to approximately 2 mm^3 and embedded in beam capsules containing fresh resin. The capsules were placed in a 60-80°C oven for one to three days. After polymerization, excess plastic was trimmed from each block to expose the tissue site.

One micron sections were made on a Sorval MT-2 (Porter-Blum) ultramicrotome for tissue orientation by light microscopy. These sections were stained with a mixture of 1% methylene blue and 1% azure II and counterstained with 2% basic fuchsin.

Appropriate structures identified in the one micron sections were then thin-sectioned. Ultra-thin sections (60-100 nm) were cut with glass knives using the Sorval MT-2 ultramicrotome and placed onto copper grids (150-300 mesh). Sections were stained for 30 minutes with 3.5% uranyl acetate in distilled water and again for 15 minutes with 0.4% lead citrate in 0.1N NaOH.

Grids were examined with a Zeiss EM 9 at magnifications from X2,000 to X28,000. Film Science Micro Image sheet film was used for photography and the negatives were printed on Kodabrome Extra-Hard, Ultra-Hard, or Polycontrast photographic emulsion. Subsequent enlargements made during printing provided electron micrographs with a final magnification range of X4,000 to X85,000.

RESULTS

Normal Placenta

A number of morphologic features of formalin-fixed placental villi were generally consistent with those which have been described in studies emphasizing various components of normal third trimester tissue fixed in glutaraldehyde and/or osmium tetroxide.

The extremely vacuolated appearance of the syncytiotrophoblast resulted from dilatation of endoplasmic reticulum (ER) (Figure 1). Rough and smooth surfaced vesicles of ER were abundant and evenly distributed throughout the cytoplasm. Endoplasmic reticulum cisternae were fairly uniform in size and often contained a flocculent material of low electron density. Although microvilli were absent from some segments, a moderate number of regularly spaced microvilli lined a large portion of the trophoblast surface. Microvilli had a variety of shapes, ranging from short forms with round or bulbous tips to elongated or rod-like forms with oval tips. A few large vacuoles resembling pinocytotic vesicles were present at the base of microvilli, occasionally extending deeper into the cytoplasm (Figure 2). Vacuoles had irregular membranes and contained electron dense granules, small vesicles, and a granular material. Their similarity to multivesicular bodies was suggested by the vesicles within. A small quantity of cytoplasmic granules included both moderately electron dense granules with a distinct limiting membrane and electron dense granules with an irregular contour. Mitochondria were scarce and had a continuous outer membrane but poorly

Figure 1. Syncytial knot in the trophoblast of a normal villus. A brush border, composed of a moderate number of microvilli (Mv), lines the membrane surface. The syncytial cytoplasm contains an abundance of dilated vesiculated ER (ER) and appears vacuolated. The clear appearance of a cytotrophoblast cell can be seen (arrow). (x 6,000)

Figure 2. Vasculo-syncytial membrane (VS) adjacent to a syncytial knot (SK) in a normal villus. Large irregular vacuoles (arrows) at the base of microvilli may be pinocytotic vesicles. Desmosomes can be seen at the junction of endothelial cells (thick arrows). (x 7,000)

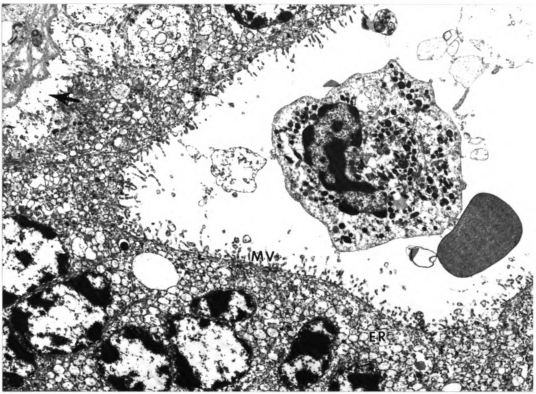

Figure 1

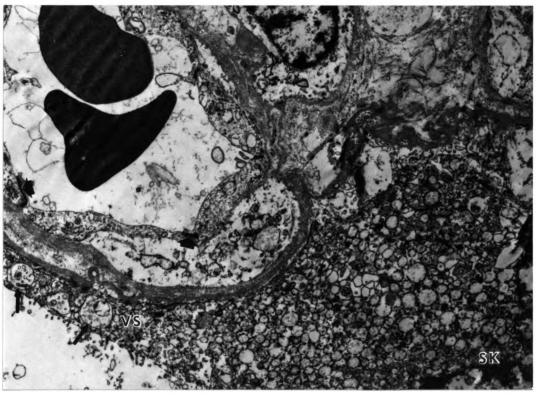

Figure 2

defined cristae which appeared to be disrupted, possibly caused by swelling. Glycogen particles and Golgi could not be detected and tonofilaments and free ribosomes were obscured by numerous cytoplasmic vesicles and vacuoles. Syncytial knots were lined by a brush border and were adjacent to vasculo-syncytial membranes (Figures 1 and 2). Intervillous bridges were also observed. Syncytial nuclei were often indented and had intact trilaminar nuclear membranes (Figure 3). They contained nucleoli and dense granular chromatin which clumped marginally. Juxtanuclear vacuoles were frequently seen (Figure 4). Numerous basal infoldings of the plasmalemma were in close proximity to a relatively thick trophoblast basement membrane which had granular inclusions. The basement membrane was moderately electron dense, homogeneous, and often convoluted (Figure 4).

Microvilli projected into intercellular spaces between syncytial cells and cytotrophoblast cells (Figures 3 and 5). Cytotrophoblast cells formed a discontinuous layer and were less electron dense than syncytial cells. Intracellular spaces and a scarcity of organelles gave many cells a clear appearance (Figures 1 and 4). Rough endoplasmic reticulum was either membranous or vacuolated and few mitochondria and granules were present. Small electron dense particles resembling ribonucleoprotein (RNP) particles were scattered throughout the cytoplasm, but Golgi and glycogen were not observed. Nuclei were oval or round with a trilaminar membrane (Figure 5). They contained nucleoli and a minimal amount of granular chromatin which clumped beneath the membrane.

Most components of the villous stroma were readily identified in formalin-fixed tissue. Terminal villi were primarily composed of sinusoidal enlargements of fetal capillaries and had few connective tissue fibers and reticulum cells. Numerous collagen fibers were seen

Figure 3. Syncytial nuclei in a syncytial knot of a normal villus. Nuclei (N) are indented and their trilaminar membranes are intact. Chromatin is clumped at the nuclear margin and microvilli (arrow) project into intercellular spaces. (x 12,600)

Figure 4. Trophoblast of a normal villus. Juxtanuclear vacuoles (JV) are associated with syncytial nuclei. The trophoblast basement membrane (BM) is thick and convoluted. Intracellular spaces can be seen (arrows) in a cytotrophoblast cell. (x 10,000)

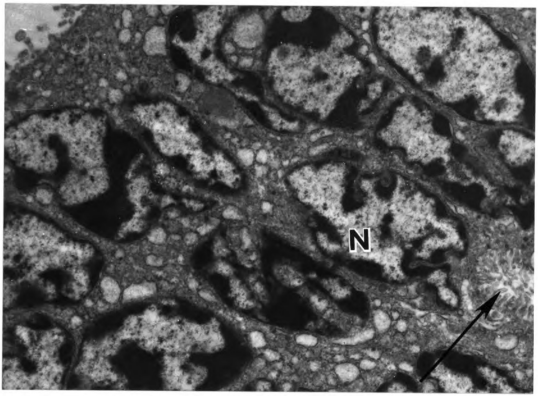

Figure 3

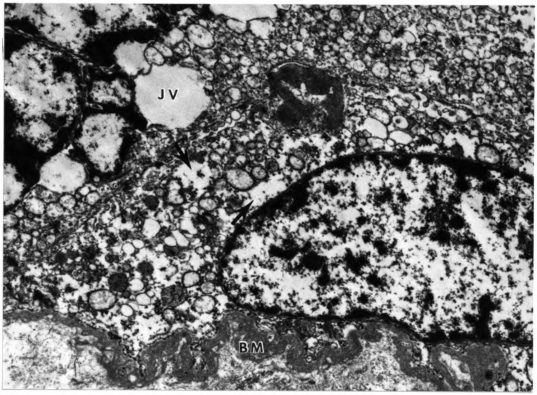

Figure 4

Figure 5. Trophoblast of a normal villus. Microvilli (Mv) project into the intercellular space between syncytial cells and cytotrophoblast cells. The trilaminar nuclear membrane (arrow) of a cytotrophoblast cell can be seen. (x 8,900)

Figure 6. Stroma of a large normal villus. Numerous collagen fibers (Col) can be seen on longitudinal and cross-section. Smooth muscle fibers (SM) are filled with myofilaments (arrow). Muscle fiber nuclei (N) seem to be twisted and contain nucleoli and marginal chromatin. (x 8,500)

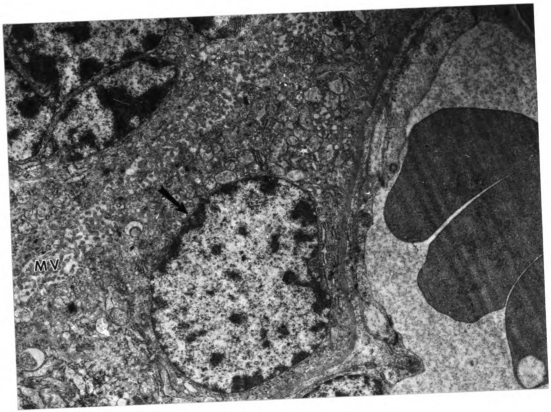

Figure 5

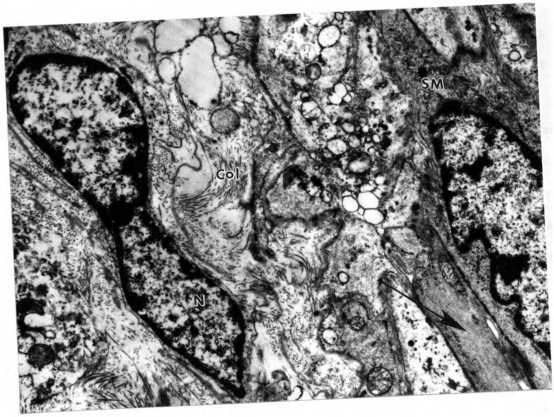

Figure 6

on longitudinal and cross-section in the larger villi (Figures 6 and 7). Long spindle-shaped muscle fibers were randomly distributed in larger villi, parallel to or overlapping adjacent muscle fibers (Figure 6). The cytoplasm of the muscle fiber was nearly completely filled with myofilaments which coursed through fusiform densities. Attachment plaques on the sarcolemma seemed to join cells and caveolae lined the inner surface of the membrane. A moderately electron dense thin basal lamina followed the contour of the cell. Muscle fiber nuclei were often constricted or appeared spirally twisted and contained nucleoli and marginal granular chromatin. Membranous ER and a few small mitochondria with misshapen cristae were present at the nuclear pole. Particles resembling RNP particles were also in this location, but glycogen particles were not apparent between the myofilaments. Fibroblasts also populated the stroma of larger villi. They were fusiform cells with long cytoplasmic processes parallel to or surrounded by collagen fibers and a moderately electron dense fibrinoid-type substance (Figure 7). Abundant cytoplasm contained a well-developed confluent network of endoplasmic reticulum, a few oval mitochondria and dense granules, and many small dense particles which may have been free RNP particles. Oval or elliptical nuclei appeared indented and contained nucleoli. Chromatin was clumped along the nuclear membrane. Intracellular spaces in fibroblasts and clear spaces in the interstitium indicated a loss of cytoplasmic sol and ground substance (Figures 7 and 8). Although nuclear membranes and surface membranes of mitochondria were intact, the plasmalemma of some fibroblasts appeared focally disrupted, demonstrating a certain degree of cytolysis.

The majority of endothelial cells were attenuated with a narrow cytoplasmic width (Figure 8). Desmosomes could be seen attaching adjacent

Figure 7. Stroma of a large normal villus. Fibroblasts (FB) are parallel to collagen fibers (Col) and fibrinoid material (F). Oval nuclei contain marginally clumped chromatin. Intracellular spaces (arrows) can be seen and cell membranes appear to be disrupted (thick arrow). (x 8,000)

Figure 8. Fetal blood vessel surrounded by collagen fibers and fibroblasts in a normal villus. Endothelial cells (En) are attenuated and attached by desmosomes (D). Cytoplasmic flaps (Cf) extend into the lumen which contains a flocculent substance. The plasmalemma of fibroblasts is disrupted (thick arrow). Note the clear spaces in the interstitium (arrows). (x 7,000)

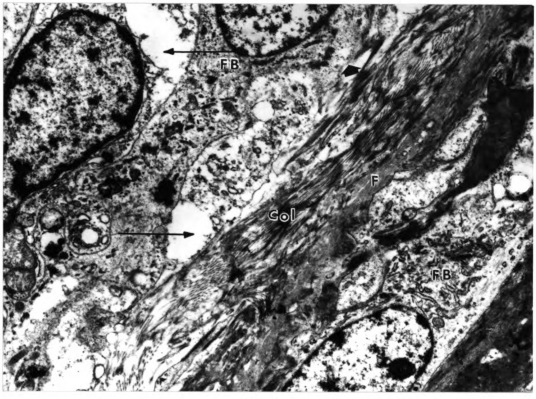

Figure 7

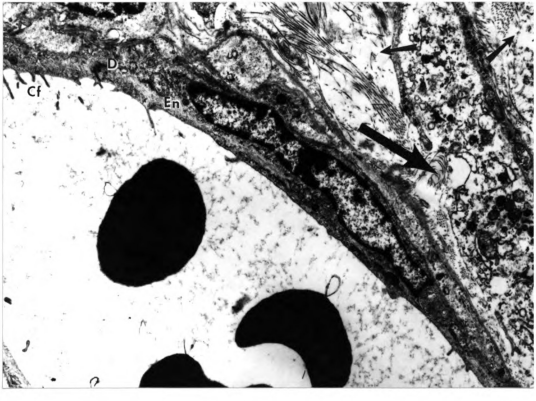

Figure 8

endothelial cells (Figures 2 and 8). Plasma membranes were continuous and cytoplasmic flaps extended into the lumen (Figures 8 and 10). Small round vesicles associated with the luminal surface of the plasma membrane were also free within the lumen and may have functioned to transport substances to the fetal circulation. They closely resembled micropinocytotic vesicles or caveolae which lined the plasmalemma. Blood vessels were surrounded by numerous collagen fibers and their lumina contained a moderately electron dense flocculent substance which was probably plasma protein (Figures 8 and 9). Endothelial cytoplasm contained many organelles. Rough ER was moderately distributed throughout the cytoplasm and numerous small electron dense particles may have been free ribosomes and polysomes. Round mitochondria had intact surface membranes and cristae. Some appeared to be swollen. Golgi complexes and dense membrane-bound granules were few in number, but glycogen was not apparent. A moderate amount of fine parallel microfibrils could be seen in between organelles (Figure 9). Nuclei were oval and contained clumped granular chromatin, much of it situated at the margin. Nucleoli were present and nuclear pores could be detected in the trilaminar nuclear membrane. Endothelial basement membranes were moderately electron dense and extremely irregular. They had several thin component layers which branched, curved, and anastomosed (Figures 9 and 10). Collagen fibers were present in the interstitium between the basal laminae of endothelium and trophoblast epithelium. The cuboidal shape of some endothelial cells suggested a state of contraction and plasma membranes at their stromal surface and basement membranes were extremely convoluted as a consequence (Figure 10). Nuclei of cuboidal cells appeared compressed. They were round or oval

Figure 9. Normal attenuated endothelial cell. Fine parallel microfibrils (Mf) and a swollen mitochondria (thick arrow) can be seen in the cytoplasm. The nucleus contains clumped chromatin. The basement membrane has several layers (arrows) and the vessel lumen contains a flocculent substance. (x 15,000)

Figure 10. Cuboidal endothelial cells in a normal villus. Nuclei appear compressed with cytoplasmic indentations. Microfibrils (arrow) are present among organelles. Several component layers of a convoluted basement membrane (thick arrow) can be seen. (x 7,300)

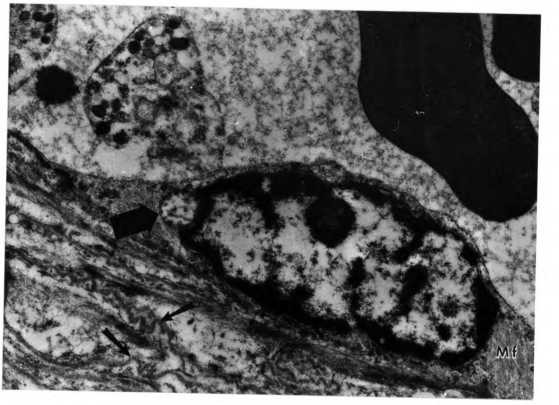

Figure 9

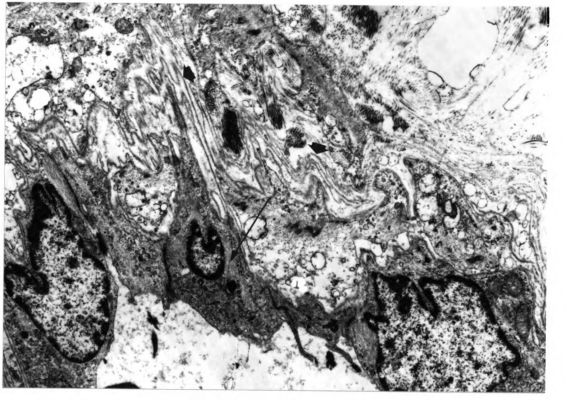

Figure 10

in shape and had deep cytoplasmic indentations. Microfibrils were seen throughout the cytoplasm of cuboidal endothelial cells.

Abnormal Placenta

The following observations of hemorrhagic endovasculitis made by light microscopy (Sander, 1980) were confirmed with transmission electron microscopy. Non-exudative necrosis of blood vessel walls was associated with extreme red blood cell fragmentation. Numerous schistocytes were present in vascular lumina and in damaged vessel walls with diapedesis and hemorrhage into the villous stroma (Figures 11 and 12). Many fragmented red cells could be seen in stroma which was often hypercellular and sometimes fibrosed. Hemorrhagic endovasculitis affected blood vessels of all sizes ranging from large chorionic stem vessels to the capillaries of terminal villi. Swollen endothelial cells were observed and obliteration of blood vessel lumina was related to cellular hyperplasia.

Ultrastructural studies elucidated some aspects of cellular hyperplasia and demonstrated additional features of connective tissue and blood vessels affected by hemorrhagic endovasculitis. The presence of inflammatory cells in damaged foci was not detected by light microscopy, but white cell fragments were seen with electron microscopy in the vicinity of fragmented erythrocytes and appeared to be of neutrophilic origin (Figure 12). Nuclei had a thick margin of dense granular chromatin and the cytoplasm contained numerous membrane-bound granules. Some granules were large, spherical, and electron dense and others were small and rod-like but variable in shape and density. These were most likely primary and specific granules, respectively.

Figure 11. Abnormal villus with hemorrhagic endovasculitis. Erythrocyte fragments can be seen in the damaged vessel wall of a fetal capillary and in the stroma (arrows). Vacuoles (V) are present in the stroma. (x 9,300)

Figure 12. Abnormal villus with hemorrhagic endovasculitis. Hemorrhage into the stroma is evident and numerous schistocytes are present. The white cell fragment (arrow) among schistocytes appears to be a neutrophil fragment. Many vacuoles (V) can be seen in the stroma. Microvilli (thick arrow) line the trophoblast and the trophoblast basement membrane (BM) is thickened. (x 5,800)

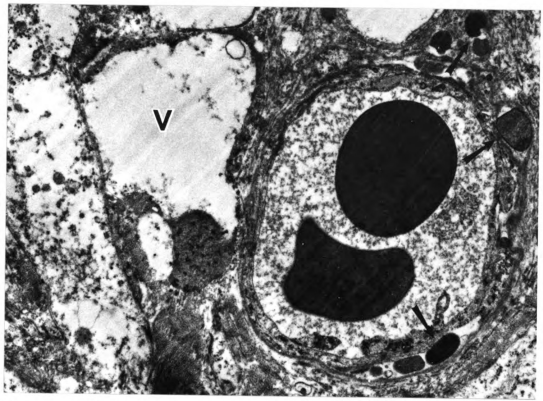

Figure 11

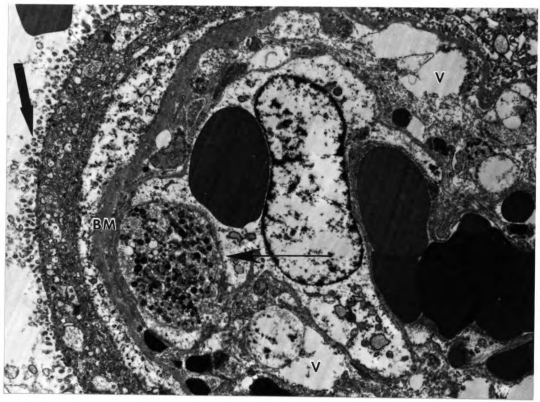

Figure 12

Blood vessel damage was often so severe in villous capillaries that the endothelium was not discernible and only a mass of schistocytes indicated the remains of a blood vessel (Figure 13). Many erythrocytes were electron lucent resulting from hemoglobin loss. The contents of these cells were granular or flocculent and occasionally included aggregates of an electron dense material which may have been hemoglobin. Red blood cells also appeared compressed and bent as they migrated through restricted openings in defective vessel walls. Red cell fragments could be seen in the cytoplasm of endothelial cells and pericytes (Figures 14 and 15). One pericyte observed contained a red cell which probably migrated through disrupted plasma membranes of both endothelium and pericyte. The pericyte resembled an endothelial cell but had dilated sacs of rough ER. Its cell sap was pale and mitochondria appeared swollen with indistinguishable cristae.

The presence of abundant cytoplasmic microfibrils was a distinctive feature of two intact capillary endothelial cells in tissue severely affected by diffuse hemorrhagic endovasculitis. Most of the endothelia in this specimen had completely disintegrated. One endothelial cell was attenuated and appeared swollen (Figure 15). The relative quantity of microfibrils in its cytoplasm seemed increased in comparison to normal endothelia and may have contributed to its swollen appearance. Microfibrils were parallel in arrangement and parallel to the longitudinal axis of the cell. They were uniformly distributed in a wavy pattern throughout the cytoplasm. There was a general absence of cytoplasmic organelles. The elongated nucleus had an atypical shape which was also characteristic of other endothelial cells in this tissue. One end of the nucleus was wide and had a deep indentation, while the opposite end was tapered. Nuclear granules were clumped and

Figure 13. Mass of schistocytes associated with a damaged blood vessel. Degenerate red cells are seen and contain a flocculent substance (Fl). (x 9,300)

Figure 14. Pericyte containing a red cell fragment (RC). Dilated sacs of ER (ER) differentiate this cell from an endothelial cell. Note the pale cell sap and the mitochondria (arrow) which appear swollen with distorted cristae. (x 13,800)

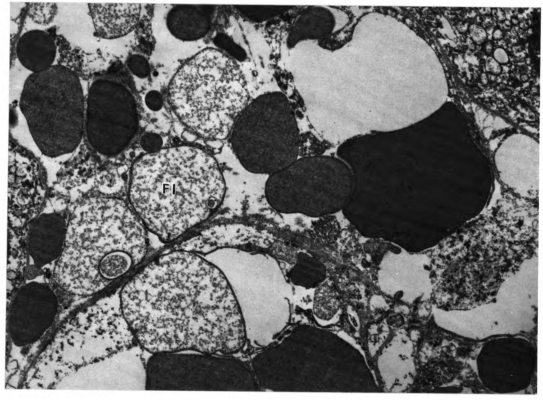

Figure 13

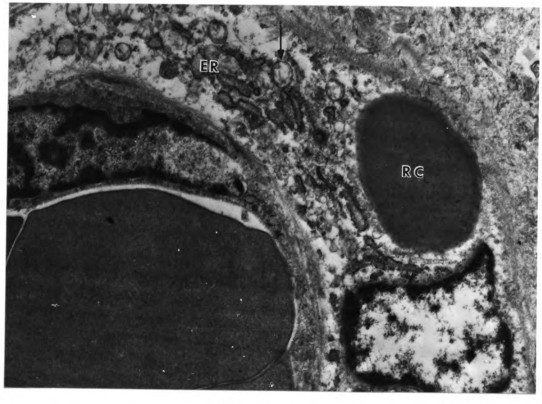

Figure 14

Figure 15. Attenuated endothelial cell associated with hemorrhagic endovasculitis. An abundance of parallel microfibrils (Mf) may have caused swelling of the cell. There is a general absence of organelles and a red cell fragment (RC) can be seen in the cytoplasm. Note the break in the plasmalemma (thick arrow). A deep indentation (arrow) can be seen in the nucleus. (x 15,000)

Figure 16. Cuboidal endothelial cell associated with hemorrhagic endovasculitis. Numerous microfibrils (Mf) can be seen in a cytoplasm which contains few organelles. Contraction of the cell may have produced the cell's cuboidal shape and the nuclear indentations (arrow). (x 32,100)

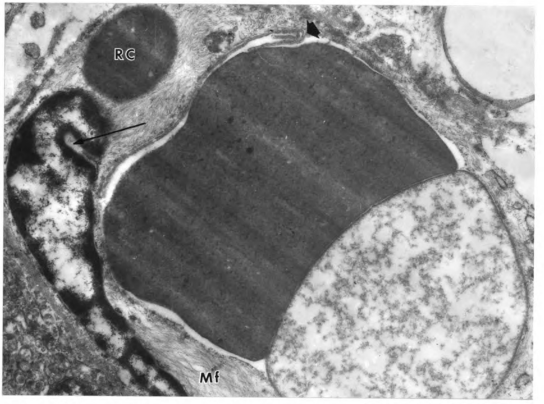

Figure 15

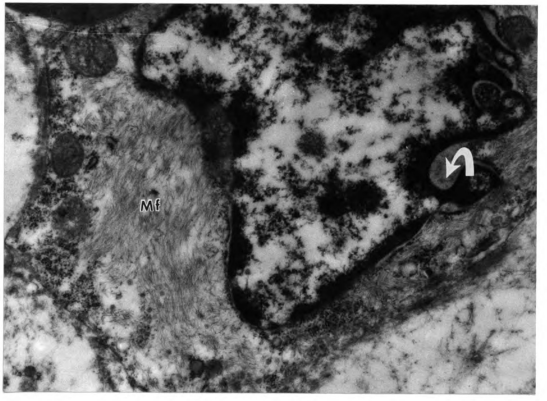

Figure 16

chromatin accumulated beneath the membrane. The plasma membrane at the luminal surface was disrupted and a strand of broken membrane loosely folded into the lumen. A red cell fragment in the cytoplasm suggested passage through this damaged site. The second endothelial cell was cuboidal in form and may have been contracted (Figure 16). Numerous microfibrils were present in a cytoplasm devoid of all organelles other than a few mitochondria and a moderate number of free RNP particles. Contraction may have produced the deep indentations which characterized its rectangular nucleus.

During light microscopic studies, Sander (1980) described medial and intimal hyperplasia associated with luminal obliteration in large stem vessels. Identification of proliferating cells in stem vessels affected by hemorrhagic endovasculitis was made by ultrastructural studies. Fibromuscular hyperplasia appeared to be a reparative process occurring in damaged vessels. Fibroblasts and smooth muscle fibers could be seen between numerous fragmented erythrocytes within the vascular lumina.

Fibroblasts were spindle-shaped cells with long cytoplasmic processes (Figure 17). Their active state was indicated by prominent nucleoli and an extensive elongated confluent network of rough endoplasmic reticulum. Dilated cisternae of ER contained a moderately dense homogeneous material. Oval indented nuclei had a thin marginal zone of dense chromatin. Chromatin appeared clumped and abundant free ribosomes were scattered in the cytoplasm. Cells contained scarce mitochondria, a few membrane-bound granules, and occasional lipid droplets. Empty vacuoles in some cells may have been sites of extracted lipid. Golgi and glycogen particles were not apparent. Fibrillar tufts with fusiform densities could be seen in the cytoplasm and were

Figure 17. Fibroblasts in the lumen of a stem vessel affected by hemorrhagic endovasculitis. Fibroblasts are spindle-shaped, contain an elongated confluent network of rough ER (arrows) and have prominent nucleoli (Nu). (x 6,300)

Figure 18. Fibroblast in the lumen of a stem vessel affected by hemorrhagic endovasculitis. Fibrillar tufts with fusiform densities (arrow) can be seen at the cell surface and thick dense irregular collagen (thick arrow) is adjacent to the fibroblast. Note the intracellular spaces and cell membrane disruption. (x 6,600)

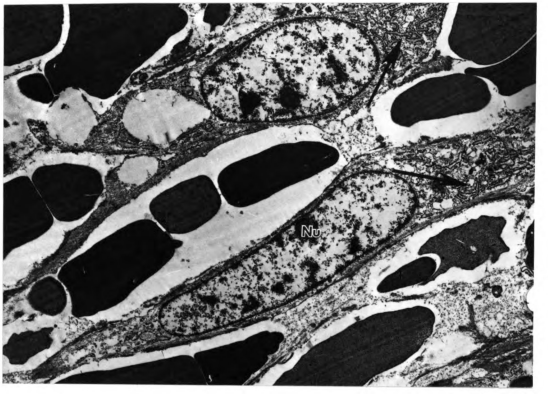

Figure 17

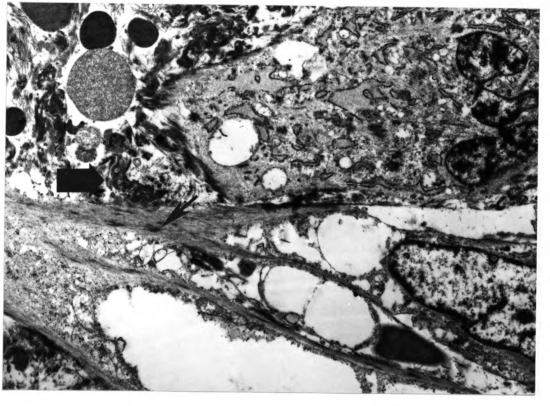

Figure 18

most often at the cell surface (Figure 18). Fibroblasts were parallel to each other and parallel to and/or surrounded by abundant collagen fibers. Fibrosis caused obliteration of the vessel lumina and was indicated by thick dense irregular collagen deposits. Intracellular spaces and discontinuous plasma membranes were also observed.

Proliferating smooth muscle cells could also be found among the many fragmented erythrocytes and collagen fibers within stem vessels (Figure 19). Muscle fibers were elongated narrow cells with tapered ends and attached to adjacent fibers. The cytoplasm was completely filled with myofilaments which coursed through fusiform densities. Caveolae and electron dense attachment plaques were present along the sarcolemma. A few mitochondria and rough ER were located at the nuclear pole but neither glycogen granules between myofilaments nor Golgi were observed. An elliptical nucleus contained dense marginal chromatin and nucleoli. The irregular nuclear outline was probably associated with the cell's elastic state.

Although major attention focused on the ultrastructure of abnormal blood vessels, some observations of connective tissue and syncytiotrophoblast associated with hemorrhagic endovasculitis were noted. A large number of collagen fibers was generally present in the connective tissue space and often invested ruptured blood vessels. A homogeneous, moderately electron dense basement membrane-like substance was often found beside collagen fibers and was usually elongated and convoluted. Erythrocyte fragments in the stroma were in proximity to thin folded electron dense threads which may have been red cell membranes released during cell rupture. Vesicles, vacuoles, and clear spaces were abundant throughout the stroma (Figures 11 and 12). A nuclear alteration was observed in one cell located in the interstitium of a

Figure 19. Smooth muscle fibers in the lumen of a stem vessel affected by hemorrhagic endovasculitis. Muscle fibers are elongated and myofilaments (arrow) and fusiform densities (thick arrow) can be seen throughout the cytoplasm. Nuclei have an irregular contour. (x 7,500)

Figure 20. Nuclear alteration in a connective tissue cell. This cell may be a smooth muscle fiber. It has myofilaments (arrow) and an irregular nuclear outline. The large eccentric vacuole (V) in the nucleus appears to have distended it and ruptured its membrane (thick arrow). (x 9,800)

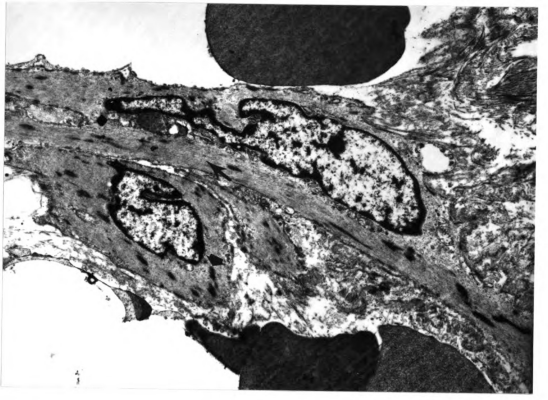

Figure 19

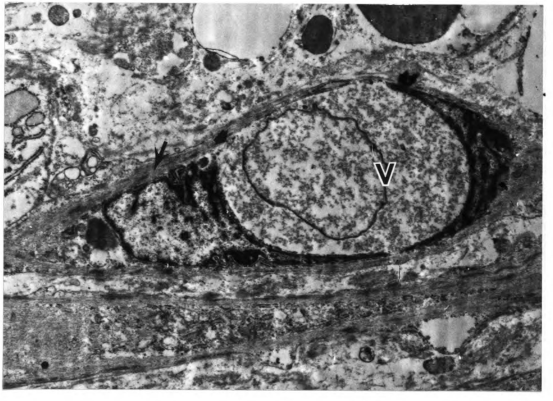

Figure 20

placental villus (Figure 20). The cell resembled a smooth muscle fiber. Its cytoplasm appeared to contain myofilaments, fusiform densities, and a few organelles at the nuclear pole. The nucleus was elliptical with dense marginal chromatin and membrane indentations. A large oval vacuole within the nucleus contained a flocculent dense substance and appeared to cause distention of the nucleus with focal disruption of its membrane.

Sander (1980) reported a frequent occurrence of chronic villitis in placentas affected by hemorrhagic endovasculitis. One placenta in this study had a histopathologic diagnosis of severe hemorrhagic endovasculitis with chronic villitis. Mononuclear cells in the stroma, villous fibrosis, and prominent syncytial knots were reported by light microscopy. Examination of affected stem vessels with the electron microscope showed a large number of neutrophils at their periphery. Neutrophils had many large electron dense primary granules and smaller specific granules of moderate density and variable shape (Figure 21). Other organelles were scarce and small dense dispersed particles may have been glycogen or RNP particles. Intracellular spaces and disruptions of the plasmalemma were observed. Nuclei had dense granular marginal chromatin and more than one lobe. The connective tissue space contained many collagen fibers, fibroblasts, and a moderately electron dense fibrinoid-type material. Intravillous fibrosis was indicated by dense thick irregular collagen deposits. The syncytiotrophoblast associated with affected stem vessels had a well-developed brush border with elongated microvilli (Figures 22 and 23). Numerous dilated vesicles of rough ER contained a flocculent substance and many other vacuoles, pinosomes, and dense membrane-bound granules could be seen in the cytoplasm. Nuclei in syncytial knots had marginally clumped

Figure 21. Neutrophils associated with a stem vessel in a villus with chronic villitis and hemorrhagic endovasculitis. These cells at the periphery of an affected stem vessel appear to be neutrophils by their primary (thick arrow) and specific (arrow) granules and multilobed nuclei. (x 7,300)

Figure 22. Trophoblast of a villus with chronic villitis and hemorrhagic endovasculitis. A well-developed brush border with elongated microvilli (Mv) lines the surface. Dilated vesicles of rough ER (arrows) are numerous in syncytial cells. Cytotrophoblast cells (CT) have a clear cytoplasm and vacuoles and granules can be seen in a thick basement membrane (BM). (x 6,500)

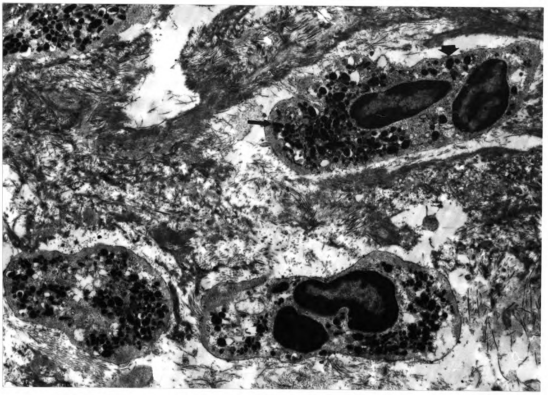

Figure 21

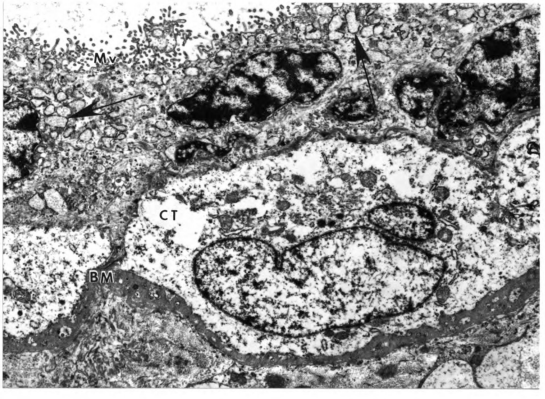

Figure 22

Figure 23. Syncytial knot in a villus with chronic villitis and hemorrhagic endovasculitis. A well-developed brush border with elongated microvilli (Mv) lines the surface. Nuclei have marginally clumped chromatin or are pyknotic (arrow). (x 7,500)

Figure 24. Degenerate portion of trophoblast associated with chronic villitis and hemorrhagic endovasculitis. Platelets (arrows) are enmeshed in fibrin-like strands and adhere to the surface of the trophoblast. (x 10,300)

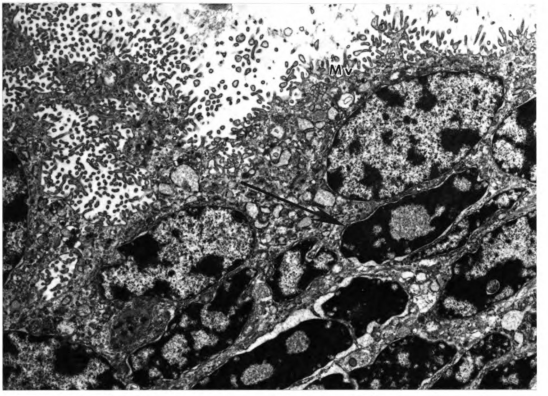

Figure 23

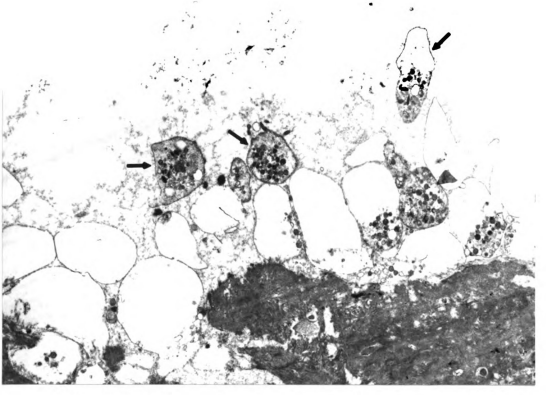

Figure 24

chromatin or were pyknotic in appearance (Figure 23). Cytotrophoblast cells had a moderate quantity of organelles within a relatively clear cytoplasm. The trophoblast basement membrane was thick and characterized by granular and vacuolar inclusions (Figure 22). Some portions of the trophoblast were degenerate and composed of fibrinoid-type material which lacked structural organization. Microvilli were absent along the surface but platelet adherence to these segments suggested the activation of clotting at the villous periphery (Figure 24). Platelets were enmeshed in a web-like network of interconnecting electron lucent strands within which platelet granules and a flocculent material could be seen.

Syncytiotrophoblast had similar morphologic features in the other abnormal placentas affected by hemorrhagic endovasculitis. Microvilli occurred along surfaces adjacent to areas of vascular damage and blunt forms with round or swollen tips were sometimes the predominant shape (Figure 12). Syncytiotrophoblast was frequently vacuolated in appearance. Dilated cisterns of ER contained a granular substance and large irregular vacuoles in proximity to microvilli contained smaller vesicles and electron dense particles. Secretory granules were present within the cytoplasm and the trophoblast basement membrane was often very thick with granular inclusions (Figure 12).

DISCUSSION

Placentas are submitted to the Registry for analysis by a large number of hospitals throughout the State of Michigan. The selection of 10% formalin as a primary fixative for placental tissue is based on a matter of economy and convenience for the hospital and its staff as well as the ease of mailing tissue from distant geographic sites. Reagent grade formalin is usually contraindicated as a fixative for ultrastructure because its methanol content (10-15%) is a protein denaturant. Studies have shown that formaldehyde can be a suitable fixative for electron microscopy if combined with an appropriate buffer system. Paraformaldehyde is more often recommended for primary aldehyde fixation and is a good preservative of ultrastructure if buffered with phosphate (Carson et al., 1972). Comparable results are obtained with tissue fixed in formaldehyde buffered with monobasic sodium phosphate and sodium hydroxide (Millonig's phosphate buffered formalin) (Carson et al., 1973). These studies of buffered formaldehyde solutions demonstrated that non-buffered 10% neutral formalin was unsatisfactory for electon microscopy because it caused considerable membrane disruption and cytolysis. It was further indicated that poor ultrastructural preservation also occurred with the more commonly used neutral formalin buffered with monobasic and dibasic sodium phosphate salts. The inferior preservative quality of neutral buffered formalin

was attributed to its acidic pH (6.85), which is below physiologic pH, and to its hypotonicity relative to plasma.

The problems encountered when evaluating formalin-fixed placentas received by the Registry with electron microscopy are threefold. First, although placentas are stored in 10% buffered formalin after receipt at the Registry, information submitted with each placenta is insufficient with regard to its initial fixation. Identification of the primary fixative is not provided. It therefore cannot be determined if tissue has been fixed in 10% neutral formalin, 10% neutral buffered formalin, or in other fixatives which are occasionally substituted for formalin. Second, the possibility of tissue autolysis is superimposed upon that of a poor quality primary fixative. Inadequate temperatures and a delay of more than two hours prior to fixation will undoubtedly have detrimental effects on ultrastructural preservation. The unpredictable circumstances associated with each delivery, especially a complicated one, can impede primary fixation. The extent of autolysis is always indeterminant and its damaging effects cannot be differentiated from those of the primary fixative. Finally, pathologic alterations cannot be accurately evaluated at a subcellular level in placentas poorly fixed in inappropriately buffered formalin. A primary objective in examining tissue pathology with ultrastructure is to assess cellular organelles in order to determine functional changes related to disease. Formalin has deleterious effects on organelles and therefore morphologic changes caused by formalin and disease become indistinguishable. Formalin may cause mitochondrial swelling, enlargement of ER cisternae, clumping of nuclear granules, an abnormal distribution of ribosomes, and damages glycogen and other labile cytoplasmic constituents. The degree of autolysis further complicates interpretation.

While it cannot be disputed that 10% neutral formalin is a poor choice of fixative for electron microscopy, it is nevertheless suitable for certain parameters of study. In the present investigation, ultrastructure proved useful for cell identification and elucidated some features of connective tissue and epithelial membranes. Although the extent of organelle preservation was vague, formalin preserved many cellular components and connective tissue elements. Ashworth and Stembridge (1964) reported surprisingly good ultrastructural results with routine surgical tissue fixed in neutral formalin. Less satisfactory results were obtained with autopsy material because autolytic changes occurred during elapsed postmortem time and induced clumping of nuclear granules, swelling and vesiculation of mitochondria, and focal disruption of plasma membranes. Useful information was derived from formalin-fixed specimens during diagnostic and investigative pathology. Electron microscopy was used to study viral and bacterial infections, glomerular lesions in the kidney, neoplasms, and pathologic lesions detected by light microscopy such as inflammation, edema, fatty metamorphosis, and atrophy.

In view of the fact that formalin is already known to affect fine structure adversely, the variable degree of preservation observed among placentas was probably most influenced by different time lapses between delivery and fixation. The extent of cytolysis should then vary with each specimen. It is possible that some tissue was also better preserved because 10% neutral formalin was buffered. Ashworth and Stembridge (1964) were not decisive about any differences between tissues fixed in buffered or non-buffered formalin.

Although 10% neutral formalin is not the ideal fixative by which to assess cellular organelles in disease, most tissue components

resisted the effects of autolysis and fixation sufficiently well for identification. Normal placentas were first evaluated in order to estimate the morphologic injury induced by autolysis and formalin.

Nearly all subcellular elements were preserved to some degree within normal cells. Postosmification of formalin-fixed placenta most likely contributed to preservation of fine structure by enhancing detail, particularly membranes, and minimizing further mechanical damage during tissue processing. Due to the denaturing effect of formalin on proteins and its inability to preserve polysaccharides, glycogen particles or rosettes were not seen. Intracellular spaces in cytotrophoblast cells and fibroblasts and clear spaces in the interstitium were particularly conspicuous. Glycogen particles should have been numerous between the myofilaments of smooth muscle fibers. Ground substance was severely affected because it is composed of glycosaminoglycans. Golgi complexes were scarce and only apparent in endothelial cells where an abundance of organelles increased their frequency of occurrence. Golgi, in general, are rarely seen because their appearance varies with the functional state of the cell, with active forms more easily detected. Mitochondria, endoplasmic reticulum, ribosomes, microfilaments, and lipid droplets were preserved, but their number, structure, and distribution could not be evaluated with certainty. The small number of mitochondria in syncytial and cytotrophoblast cells may have reflected reduced metabolic activity at term, but their scarcity in highly active cells such as fibroblasts and muscle cells suggested a reduction in number caused by autolysis and formalin. Their surface membranes were intact but distortion of cristae was probably related to these two factors. Despite the harmful effect of formalin on proteins, ribosomes were found free in the cytoplasm and

attached to ER membranes, indicating no severe effects on ribosomes or endoplasmic reticulum. The extensive dilatation of endoplasmic reticulum and small quantity of organelles, secretory granules, and pinocytotic vesicles in the trophoblast are distinct features of placental senescence and unlikely to be related to formalin fixation. Microfilaments were well-preserved. Myofilaments in smooth muscle fibers and microfibrils in endothelial cells were prominent.

Nuclear membranes were consistently intact, as indicated by their continuity and layered appearance. Autolytic changes superimposed by formalin fixation were more apparent within the nucleus and caused clumping and condensation of nuclear granules. Membrane disruption resulting from autolysis and/or formalin fixation was more frequently seen in plasma membranes of cells in the interstitium. Plasma membranes of the trophoblast demonstrated no apparent damage and the shape and quantity of microvilli were consistent with observations of tissue preserved by superior fixatives.

Collagen fibers and fibrinoid material in the stroma and basement membranes appeared to be unaffected by formalin. Cell shapes and characteristic features were maintained adequately for cell identification. The flocculent or granular contents of vascular lumina suggested the presence of plasma proteins.

Cuboidal endothelial cells were an interesting finding. Their cuboidal shape may have been caused by placental contraction associated with uteroplacental circulatory regulation. Their prominent cytoplasmic microfibrils may have functioned to support the cells under mechanical pressure caused by contraction. This is contrary to the observations of Terzakis (1963), who reported a less distinct appearance of microfibrils in contracted cells and proposed a contractile function for them.

Ultrastructural interpretation of formalin-fixed placenta was more ambiguous in abnormal tissue. Two additional factors influenced interpretation of ultrastructure in placentas with hemorrhagic endovasculitis. Pathologic processes and normal placental senescence produce effects on fine structure which are analogous to those caused by autolysis and formalin fixation. It is therefore difficult to correlate cellular changes with disease when they are likely to result from autolysis, fixation, or a normal aging process.

Pathologic changes in cells may include dilatation of ER cisternae and detachment of ribosomes, mitochondrial swelling with distortion of cristae, cell fusion, fragmented Golgi, contraction of microfilaments, and a decrease in density of the cell sap. Nuclei may have deep cytoplasmic indentations or contain vesicles, vacuoles, virus particles, or fibrillar structures. Anoxia or ischemia results in clumping of chromatin caused by reduced cell pH with lactate accumulation. Clumping is also associated with virus-cell interactions. Swelling or hypertrophy of the cell and a reduction in organelles and glycogen can occur with disease. Breaks in the plasmalemma indicate irreversible injury.

Features of placental senescence are often difficult to distinguish from those of disease or fixation. Changes in cellular organelles generally reflect decreased metabolic activity and hormone production. The trophoblast has fewer organelles and more dilated ER with numerous cisternae. Syncytial cell membranes fuse. Syncytial necrosis occurs to some extent and autophagic vacuoles may be present. Syncytial knots are considered by many to be an aging phenomenon and myelin figures represent degenerative nuclei. Basement membranes are often thickened,

collagen fibers may be abundant, and fibrinoid deposition may be related to cellular degeneration and fibrin accumulation.

The confirmation of major morphologic features of hemorrhagic endovasculitis with electron microscopy is an important aspect of this study. Extremely minute erythrocyte fragments in the villous stroma and blood vessel lumina and walls demonstrated extensive injury to red cells. Fragmentation may have been caused, in part, by proteolytic and lipolytic enzymes released from necrotic vessel walls. Conformational changes of red cell shapes during passage through focal disruptions in vessel walls verified diapedesis in necrotic areas. Diapedesis with hemorrhage into the villous stroma was also observed by Okudaira et al. (1966) during electron microscopic studies of placentas from diabetic mothers. Nearly complete destruction of the fetal vasculature occurred with diffuse hemorrhagic endovasculitis and undoubtedly influenced fetal survival.

The presence of white cells in areas of vascular damage was an unexpected observation because this vascular lesion appears to be non-inflammatory by light microscopy. Lysosomal and specific granules identified these cells as neutrophils. Inability to detect neutrophils by light microscopy was probably due to their infrequent occurrence. While it is possible that their presence was associated with chemotaxis, it is more likely that they were released from ruptured blood vessels.

The abundance of microfibrils in two endothelial cells was an interesting observation. Numerous endothelial microfibrils have been reported in placentas associated with gestosis (Dec et al., 1977), maternal diabetes mellitus (Lister, 1965; Okudaira et al., 1966; Widmaier, 1970), erythroblastosis (Zacks and Blazar, 1963), and

maternal pre-eclampsia (Jones and Fox, 1980). In placentas with hemorrhagic endovasculitis, the swollen appearance of endothelial cells observed with light microscopy may have been caused by an increment in microfibrils. Microfibrils completely filled the cytoplasm of the attenuated cell and appeared to distend it. The absence of other cellular organelles may have related to a number of factors. Organelles could have been poorly preserved or an outflow of organelles may have occurred through the cell membrane, ruptured by fixation, autolysis, or a pathologic process. Davies and Glasser (1967) also observed a small quantity of organelles in placental endothelia after fetal death. Disease could have reduced most cellular organelles while concurrently stimulating proliferation of microfibrils. Nuclear indentation may have been another feature of disease and clumping of nuclear granules may have resulted from fixation, autolysis, anoxia, ischemia, or virus-cell interaction.

Intracellular microfibrils are thought to have a contractile or supportive function. Actin has been identified in placental endothelia (Faulk and Johnson, 1977) and may be related to microfibrils allowing contractility of blood vessels lacking a muscular media. Microfibrils may cause dilatation of capillary sinusoids in terminal villi to extend fetal vessels closer to the maternal blood space for efficient exchange. Hypoxia resulting from destruction of fetal blood vessels in hemorrhagic endovasculitis may stimulate proliferation of microfibrils to further dilate remaining capillaries and expedite oxygen transfer.

Alternatively, an increase in microfibrils may reflect cellular degeneration with hemorrhagic endovasculitis. Vacek (1969) proposed

that cellular filaments might be fibrous protein formed during regressive changes.

Microfibrils are prominent in attenuated and contracted normal endothelial cells. They may provide mechanical support to sustain the pressures of placental contraction associated with uteroplacental circulatory regulation. Microfibrils in the abnormal attenuated cell may have increased in response to external pressure created by a hypercellular stroma. Contraction of microfibrils often occurs with disease and might have produced the cuboidal shape of the second abnormal endothelial cell.

Microfibrils are associated with intracellular movements, as in phagocytosis, and general cell motility. A phagocytic ability of endothelial cells has been proposed and it is possible that the intracellular red cell fragment observed in the attenuated cell was ingested.

In general, the purpose of endothelial microfibrils remains unresolved. They may be contractile or supportive in function, or may participate in cell motility. Hammersen (1980) did not observe an increase in cellular height with possible shortening of microfibrils and favored an adhesive and supportive function for them based on their location near the cell surface. While providing mechanical support for placental endothelia, it is possible that microfibrils are also contractile because they are homogeneously distributed and there appears to be an increase in cellular height with cuboidal cells. Although cuboidal cells may result from placental contraction, they might also occur upon contraction of microfibrils.

The cause of hemorrhagic endovasculitis is unknown, although infection has been considered (Sander, 1980). It is interesting to speculate on a possible correlation between the large number of

microfibrils in observed endothelia and maternal hypertension. Medical histories of mothers are submitted with placentas but provide insufficient information and therefore maternal hypertension is indeterminate. Erythrocyte fragmentation could be caused by hypertension. Hammersen (1980), in a review of endothelial filaments in the microvasculature, described numerous filaments in proliferating endothelia and in endothelia subjected to the mechanical stress of high blood pressure. In affected placental endothelia, microfibrils may have been prominent due to a reduction in other organelles, but they may have proliferated in response to maternal hypertension. Dec et al. (1977) reported an abundance of microfilaments in placental endothelia associated with gestosis (hypertension, proteinuria, and edema). They observed endothelial damage from high blood pressure which resulted in intraluminal thrombus formation. Thrombi have been implicated in mechanical damage to erythrocytes (Dacie, 1967; Bull et al., 1968) and acutely progressive malignant hypertension in adults is associated with red blood cell fragmentation. Intravascular thrombi were described in placental blood vessels affected by hemorrhagic endovasculitis (Sander, 1980) and may be related to erythrocyte fragmentation and hypertension.

In large stem vessels, medial and intimal proliferation was reported by light microscopy (Sander, 1980). In tissues examined with the electron microscope, proliferating cells in stem vessels were identified as fibroblasts and smooth muscle fibers. Fibromuscular hyperplasia appeared to be a reparative process which occurred in response to vascular damage. Luminal obliteration resulted from deposition of dense fibrous collagen. Dilated ER cisternae in fibroblasts contained a substance which may have been a protein precursor of collagen.

Sclerosis with thickening of vessel walls may also have resulted from intrauterine fetal death with continued viability of the placenta. Davies and Glasser (1967) described sclerosis of large stem vessels in fetal death with devascularization and fibrosis of the villous stroma. Theuring (1968) also reported fibrous obliteration of vessels with intrauterine death and persistence of the placenta. Chromatin clumping, organelle reduction, intracellular spaces, and cell membrane disruption in proliferating cells indicated the adverse effects caused by autolysis and/or formalin-fixation.

Numerous collagen fibers in connective tissue were particularly noticeable in terminal villi which normally contain only a few fibers at the periphery of dilated capillary sinusoids. Placental villi become fibrotic with normal placental regression, but collagen deposition is accentuated with prolonged pregnancy (Kemnitz and Theuring, 1974; Jones and Fox, 1978a; Thliveris and Baskett, 1978), fetal death (Davies and Glasser, 1967), maternal-fetal rhesus incompatibility (Jones and Fox, 1978b), maternal hypertension (Dec et al., 1977; Thliveris and Speroff, 1977), and maternal pre-eclampsia (Jones and Fox, 1980). The homogeneous substance among collagen fibers may have been another form of collagen. It resembled basement membrane, which is thought to be partially composed of collagen Type IV. Basement-membrane-like substance has been described in placentas following fetal death (Davies and Glasser, 1967) and in placentas from diabetic mothers (Okudaira et al., 1966). The presence of abundant collagen in placental villi affected by hemorrhagic endovasculitis suggests intense scarring following vascular damage. Eventual villous fibrosis could also occur after intrauterine death with a drop in fetal blood pressure.

Vesicles and vacuoles observed in the connective tissue are also a feature of placentas associated with prolonged pregnancy (Jones and Fox, 1978a), maternal diabetes mellitus (Okudaira et al., 1966), and maternal pre-eclampsia (Jones and Fox, 1980). Clear spaces in the interstitium probably resulted from poor preservation of ground substance.

The nuclear alteration in an apparent smooth muscle fiber was an unusual finding. It may have been an artifact produced by tissue preparation. The appearance of the intranuclear vacuole resembled that of a degenerated blood cell because it contained a flocculent substance. A degenerated red cell may have been compressed against the nucleus. The vacuole appeared similar to those of unknown significance which characterize diseased cells (Trump and Jones, 1978) or some normal cells. Vesicles containing an amorphous precipitate often dilate the nuclei of placental macrophages or Hofbauer cells (Boyd and Hamilton, 1970). This nuclear inclusion may also have been related to a virus-cell interaction if hemorrhagic endovasculitis is virus induced.

Non-specific chronic villous inflammation, or villitis of unknown etiology, was reviewed by Russell (1980). Acute inflammation is followed by post-inflammatory scarring and is related to retarded intrauterine growth and perinatal mortality, a possible consequence of placental damage. Russell speculated on an infectious etiology for unexplained chronic villitis. Its frequent association with hemorrhagic endovasculitis suggests an infectious cause for this alteration also (Sander, 1980).

Ultrastructure demonstrated numerous fibroblasts and collagen fibers in the placenta affected by both chronic villitis and hemorrhagic endovasculitis, which correlated with villous fibrosis observed by light

microscopy. With electron microscopy, polymorphonuclear cells were detected at the periphery of fetal stem vessels affected by hemorrhagic endovasculitis. They may have been fetal inflammatory cells infiltrating the vessel walls. In villitis of unknown etiology, Russell described acute fetal vasculitis with infiltration of stem vessels by inflammatory cells. These cells were implicated in rupture of the vessels and destruction of both vessels and villous architecture. It is therefore possible that chronic villitis predisposes to hemorrhagic endovasculitis in certain instances. Inflammatory cells may cause primary damage to stem vessels. Red blood cells could subsequently fragment upon contact with fibrin in damaged endothelium. Autolysis and/or poor preservation of neutrophils were indicated by cell membrane rupture and intracellular spaces. Platelet adherence to degenerated segments of trophoblast suggested fibrin formation in these areas. Platelets appeared to be enmeshed in fibrin-like strands and may have been associated with intervillous clotting. Russell (1980) reported dissolution of the trophoblast, intervillous thrombi, and adhesion of adjacent villi by fibrin.

Prominent syncytial knots were observed with electron microscopy in this same placenta. Chromatin clumping and pyknosis were difficult to assess and may have been caused by autolysis, formalin-fixation, a pathologic process, or placental aging. Subcellular analysis of villous cells could not be determined with any certainty. Syncytial knots generally increase with placental senescence, but their increased number may have reflected ischemia and hypoxia in the tissue (Jones and Fox, 1977). Ischemia and hypoxia could also have caused condensation of nuclear chromatin. Jones and Fox (1977) described degenerative changes in nuclei of syncytial knots. They proposed that sequestration of degenerated nuclei compensated for inadequately perfused villi by

providing more efficient exchange sites in adjacent areas of trophoblast without nuclei. The syncytial knots associated with chronic villitis and hemorrhagic endovasculitis probably related to hypoxia and ischemia caused by vascular damage and cessation of fetal circulation following intrauterine death.

Fibrinoid-type substance observed within villi is also a feature of normal placental aging but may be increased with prolonged pregnancy (Thliveris and Baskett, 1978), maternal hypertension (Thliveris and Speroff, 1977) and maternal diabetes (Lister, 1965). The resemblance of placental fibrinoid to hyalin and amyloid (Boyd and Hamilton, 1970) suggests that its large quantity in the placenta with chronic villitis and hemorrhagic endovasculitis may have resulted from the inflammatory process. Fibrinoid appears similar to fibrin and has been described as a filamentous network enclosing platelets (Boyd and Hamilton, 1970). Fibrinoid deposits form on the trophoblast during gestation. Platelets and the interconnecting strands on the syncytial surface could also have been fibrinoid.

Some observations of trophoblast in placentas with hemorrhagic endovasculitis were similar to those reported in placentas with other complications. Trophoblast overlying sites of vascular damage contained secretory granules and were often lined by a brush border associated with pinosomes and vacuoles. There appeared to be a retention of transport and secretory activities in these areas despite stromal injury. Davies and Glasser (1967) reported microvilli and pinocytotic vesicles in viable trophoblast two weeks after fetal death. They proposed that transport functions continued in the trophoblast when villi became fibrosed. Elongated microvilli along some segments of trophoblast in placentas with hemorrhagic endovasculitis resembled

microvilli of early gestation which are associated with considerable absorption and resorption. They may have proliferated in response to ischemia and hypoxia caused by vascular damage. Proliferation of microvilli in foci adjacent to necrosis has been observed in prolonged pregnancy (Jones and Fox, 1978a).

Evaluation of trophoblast cells for pathologic changes was not possible because several variables could have affected cellular organelles. The large number of dilated vesicles of ER in syncytial cells may have indicated a pathologic change. Vesicular ER also increases with normal placental aging. Dilatation of ER could have resulted from ischemia caused by the vascular lesion and/or fetal death or by a prolonged time lapse between cessation of maternal circulation and tissue fixation. Schweikhart and Kaufmann (1977) reported cystic dilatation of ER when the "time of ischemia" exceeded 7-8 minutes. They also observed dilatation of ER with hypoxia. Dilated ER has been described in numerous conditions affecting placentas (Davies and Glasser, 1967; Widmaier, 1970; Thliveris and Speroff, 1977; Kemnitz and Theuring, 1974; Dec et al., 1977; Thliveris and Baskett, 1978; Jones and Fox, 1978a,b).

The clear appearance of cytotrophoblast cells could not be attributed to either fixation or disease. Cytoplasmic sol may not have been preserved by formalin or may have appeared pale due to disease or cell death. Cytotrophoblast cells are generally resistant to hypoxia and are thought to replenish degenerating syncytial cells in disease because they proliferate in a number of abnormal conditions (Lister, 1965; Davies and Glasser, 1967; Thliveris and Speroff, 1977; Jones and Fox, 1978a,b; Jones and Fox, 1980). This suggests that cytotrophoblast cells associated with hemorrhagic endovasculitis were

most likely affected by formalin because they are capable of proliferating under the hypoxic conditions created by vascular damage. Thick trophoblast basement membranes may have been a product of active cytotrophoblast cells (Jones and Fox, 1978b). However, alterations in trophoblast basement membranes could not be assessed because they were often thick in both normal and abnormal placentas.

It seems reasonable to conclude that the use of ultrastructure for examining pathologic changes in placentas with hemorrhagic endovasculitis has limited value if 10% neutral formalin is used as a primary fixative. Some major ultrastructural features of cells are preserved sufficiently well to clarify their identity. However, characteristic changes which reflect altered function attributable to disease cannot be determined at a subcellular level. Improved communication with participating hospitals is needed in order to establish acceptable fixation procedures if electron microscopy is to be employed for placental analysis. Cooperation should be requested from hospital staff so that the effects of autolysis due to delayed fixation can be minimized. Hospital personnel must also be advised to use phosphate buffered paraformaldehyde or neutral formalin buffered with monobasic sodium phosphate and sodium hydroxide as a primary fixative. Proper care of specimens initially will reduce the effects of autolysis and formalin fixation which are indistinguishable from pathologic alterations.

An infectious etiology for hemorrhagic endovasculitis has been considered (Sander, 1980) and a virus could be the causal agent. Although viruses are preserved in formalin-fixed tissue and might be detected by ultrastructural examination, cultural identification is strongly recommended. While a particular family of virus could be identified by size and morphology with ultrastructure, culturing of

the maternal reproductive tract should be performed in order to characterize the strain(s) antigenically.

Virus particles might be detected in affected placentas and confused with virus particles known to occur in normal placentas. C-type virus particles belong to the RNA group of viruses and have been associated with tumors and cancer (Gross, 1976). C-type particles have been localized in trophoblast tissue of placentas from humans and other primate species (Kalter et al., 1973a,b). Applied virology will be needed to identify any virus present and to distinguish C-type particles from other RNA viruses, such as rubella, and DNA viruses if placentas with hemorrhagic endovasculitis do, in fact, contain viruses.

SUMMARY

The value of using 10% formalin-fixed human placenta for studies by immunofluorescence microscopy and transmission electron microscopy was determined.

Immunofluorescence microscopy appeared to be impractical for localizing immunoglobulins in normal 10% formalin-fixed placenta as well as for determining increased deposition of immunoglobulins in abnormal fixed tissue, such as that affected by hemorrhagic endovasculitis. Antigenicity of immunoglobulins appeared to be altered by formalin and much non-specific background fluorescence occurred. These factors interfered with detection and interpretation of specific fluorescence. Specific fluorescence of blood vessel lumina and endothelia was associated with antiserum to IgG but other sites of globulin, complement and fibrinogen were not detected despite trypsinization, which seemed to be ineffective in restoring the antigenicity of these proteins. Proteolysis and increasing dilutions of fluorescein conjugates had minimal effect on reduction of background fluorescence which obscured specific fluorescence. It was concluded that determining increased deposition of immunoglobulins in diseased placenta could be better accomplished by quantitation of antibodies eluted from fresh tissue.

The use of transmission electron microscopy for analysis of 10% formalin-fixed placenta had limited usefulness. Formalin fixation and

autolysis resulted in loss of ground substance, cell membrane disruption and poor preservation of subcellular components. Ten percent neutral formalin appeared to be suitable for certain parameters of study only. Preservation of major cellular features enabled cell identification and some connective tissue elements were also maintained.

Ultrastructural interpretation of tissues affected by hemorrhagic endovasculitis was ambiguous because subcellular changes could not be attributed to disease, senescence, fixation, or autolysis. However, major morphologic features reported by light microscopy were confirmed with electron microscopy. Proliferating cells in affected stem vessels were also identified and some features of connective tissue and trophoblast were elucidated. Results indicated that improved fixation procedures are needed for adequate preservation of fine structure if subcellular analysis is to be employed for efficient evaluation of formalin-fixed placentas.

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