HEPATITIS VIRUS MARKERS OF THE POPULATION IN THE SOUTHERN PLAIN REGION OF HUNGARY; PERINATAL AND/OR TRANSPLACENTAL TRANSMISSION OF VIRUSES

PhD THESIS

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Összefoglalás

MAGYARORSZÁG DÉLALFÖLDI RÉGIÓJÁBAN A LAKOSSÁG HEPATITIS VÍRUS MÁRKEREI; AZ EMBERI VÍRUSOK PERINATÁLIS ÉS/VAGY TRANSZPLACENTÁRIS ÁTVITELE

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Magyarországon országos megelőző program indult a hepatitis B vírus (HBV) perinatális átvitelének a csökkentésére 1995-ben. Az országos "kötelező" HBV védőoltás bevezetése 2001-ben kezdődött meg.

A vizsgálatok a hepatitis vírus márkerek meghatározásával kezdődtek a délalföldi régió lakosságában. Ezeket kiegészítette a HBV altípusok és a hepatitis B vírus tumorkeltő "X" fehérjéjének és a specifikus ellenanyagok vizsgálata. Az adatokat később a vakcinációs kampányok hatékonyságának az ellenőrzésére lehet majd felhasználni.

A közelmúltban ellentmondó eredmények jelentek meg a vírusok perinatális és transzplacentáris átvitelére vonatkozóan. Ezért a munkát kiegészítettük a klinikailag egészséges terhesek szülés előtt levett amnionfolyadékainak molekuláris vizsgálatával.

Az eredmények szerint a védőoltások és megelőző intézkedések csökkenteni fogják a HBV előfordulását a 20 év alatti korcsoportban (<1.0 %). Megfigyeltük, hogy az X-antigén és a specifikus ellenanyagok valószínüleg immunkomplexeket képeznek, hasonlóan a többi vírusfehérjéhez. A szüléskor vett amnion folyadékok egyharmada tartalmazott herpesvírus 4, 5, 6, 7 és 8 DNS-t. Alacsony koncentrációban endotoxint és papillomavírusokat is ki lehetett mutatni. Az anyai vér és az amnionfolyadék vírustartalma arra utalt, hogy a transzplacentáris vírusátvitel egyenlőtlenül történik.

Summary

HEPATITIS VIRUS MARKERS IN THE POPULATION OF THE SOUTHERN PLAIN REGION OF HUNGARY; PERINATAL OR TRANSPLACENTAL TRANSMISSION OF HUMAN VIRUSES

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Nation-wide programmes have been initiated in Hungary for the prevention of perinatal transmission of hepatitis B virus (HBV) in 1995 and mandatory vaccination has been initiated in 2001.

The prevalence of hepatatis virus markers have been determined in the Southern Plain Region of Hungary. The presence of oncogenic X-protein (HBxAg), specific antibodies and genotypes of hepatitis B virus were also examined. These data can be used later for the control of efficacy of preventive programmes.

Recently conflicting results have been published on the perinatal and transplacental transmission of viruses, therefore a systematic molecular examination of healthy pregnants and amniotic fluids taken at term were carried out.

Results confirmed that the preventive measures will reduce the occurrence of HBV prevalence to very low level (< 1 %) in the age group below 20 years. It has been observed, that the X-antigen and antibodies form probably immunocomplexes in the plasma similar to other hepatitis B viral proteins. One third of the amniotic fluid amples at term were found to contain DNA of herpesvirus types 4, 5, 6, 7 and 8. Low levels of endotoxin and papillomaviruses could be also detected. The comparison of virus content of maternal blood and amniotic fluid suggested protracted transplacental transmission of viruses.

1. Introduction

1.1 Perinatal transmission of hepatitis B virus

Hungary is a country in the middle of the Carpathian basin with low prevalence of all classical hepatitis viruses (131).

Intrafamily transmission of hepatitis B virus (19, 85, 223). and the possibilities of non-parenteral (90, 94) and perinatal transmissions (61, 137, 182) have been recognized early. The clinical course and the acute and chronic consequences of the disease have been published and reviewed by many authors (5, 25, 36, 97, 88, 129, 181, 192, 231, 232).

The possibility of perinatal transmission and the epidemiological role of chronic carriers have been also discussed by many researchers (4, 30, 70, 79, 111, 130, 151, 188, 201, 207, 208, 209). Differences in the frequency of perinatal transmission in several geographic regions and ethnic groups have been also described (53).

The possibility of prevention of perinatal transmission of hepatitis B virus have been proposed early (20, 166, 225). Years elapsed, however, until the first campaigns for the prevention of perinatal transmission have been initiated (6, 27, 103, 105, 117, 121, 150, 171, 193, 194, 203, 204, 205, 210, 214, 224, 234). A preliminary economic evaluation of the Hepatitis B prevention in Europe has indicated considerable savings in contrast to the expenses of therapy and loss of life expectancy (217, 224).

The first epidemiological evidence of “endemic” hepatitis in Hungary was found in 1948 (22). The first Hungarian preventive campaign was

initiated in 1980 in the Veszprém county (105). The first country-wide programme for the prevention of perinatal transmission has started in 1995 (195). During the first year of the programme 624 HBV carrier mothers were identified from 114,095 pregnant women.

In 1995 a nation-wide hepatitis B eradication programme has been initiated in the laboratories of the NPHMOS (National Public Health and Medical Officer Service), about 900,000 pregnant women have been tested for the presence of HBsAg (hepatitis B surface antigen) and about 4500 of them have been found to be chronic carriers. All of the neonates of the chronic carriers have been preventively vaccinated with hyper-immune gamma globulin and recombinant HBsAg vaccine simultaneously injected into two separate sites (175). The control examinations performed 2 years later have revealed that less than 1.0 per cent of the children remained HBV carriers in contrast to the frequency (25 to 45 %) measured before the initiation of the eradication campaign (144). All professionals have been convinced that such a high rate of effective preventive vaccination can be only achieved if no transplacental transmission of the virus may occur.

Recently, however, it has been published that blood samples of 41 % of healthy neonates born at term from HBV-carrier mothers had been positive for HBV-DNA. Both vaginal secretion and amniotic fluid have revealed HBV-positivity of 47.5 and 57 %, respectively (230). These findings have been considered as the discovery of transplacental transmission of HBV at the very end of the pregnancies. Unfortunately in a “low HBV prevalence” country it is not possible to organise the collection of amniotic fluid samples from HBV carrier pregnants.

1.1.1 I*mmune-response of virus carrier persons*

HBV-carrier blood donors can be identified since the early seventies by the detection of HBsAg in their blood plasma or serum (27, 53, 83, 166). In a considerable proportion of perinatally infected HBV-carriers immunotolerance to the HBeAg develops (225) or the formation of immunocomplexes render the sera negative for both HBeAg and antibodies. The HBeAg can be detected very frequently in the blood and some secretions of chronic HBV carriers. The immunotolerance, however, tends to be eliminated with time and in the majority of elderly HBV-carriers anti-HBe antibodies appear (91, 144). Immunotolerance has been never seen to develop against the "core" antigen of the virus (HBcAg). The majority of HBV-carriers are anti-HBc seropositive (144, 216). The possible reason for this difference might be the low probability of the interaction of internal "core" protein with fetal dendritic cells, which are converted to the $IL10^{high}$ $IL12^{absent}$ phenotype with tolerogenic potential (120). The envelop of HBV, however, carrying the HBsAg cannot avoid the contact with the high number of tolerogenic DC preventing the development of an immune response i.e. that of the clinical disease, too (36, 37, 120). The non-cytocydial elimination of viruses has been also discovered recently (80, 81, 82). Tumour necrosis factor, interferon gamma and other cytokines are probably responsible for the repression of viral DNA replication and seroconversion of the chronic symptomless carrier persons.

Until the beginning of this work, there was no serological test available which could test the immunological nature of HBxAg the oncogenic protein of HBV. The cloning of the *HBx*-gene into expression vector (128) made it

possible to develop serological methods for the comparative measurement of specific antibodies and HBx antigens in the serum of HBV-carriers, in acute hepatitis patients, and in patients suffering from chronic active hepatitis (148, 149).

The immunological character of the X-protein of HBV might contribute to the induction of hepatocellular cancer associated with chronic hepatitis (129).

1.2. Problem of perinatal and transplacental transmission

Differentiation of transplacental transmission of viruses from transmission during delivery has been documented in the case of HIV being transmitted accidentally by transcytosis well before delivery. Tumor necrosis factor-alpha (TNF-α) have been shown to increase transcytosis (152).

On the basis of the genetic evidences supporting the possibility of transplacental tarnsmission of HIV, one may suppose, that other latent or persistant viruses of the pregnant women can be transmitted accidentally through the placenta. About 2 per cent of the HIV-positive pregnant women infect their fetus through the transplacental mechanism. Thus one might suppose that the non-preventable 1 per cent of hepatitis B infected neonates have been also infected by the transplacental route.

Acute infections of pregnant women were shown to cause *in utero* transplacental infection of the fetus with human cytomegalovirus (HCMV), varicella zoster virus (VZV), or rubellavirus. In the case of HCMV, however, vertical transplacental infection in the presence of maternal antibodies, infection during delivery, and infection in connection with breast feeding have been described, too (141). Specific antibodies to HCMV were also suggested

to inhibit or enhance maternal-fetal transmission of the virus due to transcytosis, Fc-mediated pinocytosis (125, 138) or under pathologic condition (136). The hepatitis B carrier state or chronic infection has been shown to contribute to HSV infection, too (8), although the mechanism is not clear (69, 74, 75).

1.3. Presence of viral nucleic acids in the blood, urine of neonates and in fetal tissues

Newborn screening cards with dried droplets of neonatal blood in South-Australia had been collected between 1986 to 1999 (72). The historical samples have been tested for the presence of Epstein-Barr virus (EBV), Kaposi's sarcoma herpesvirus (KSHV), HCMV and human herpesviruses 6 and 7 (HHV6 and HHV7) DNA using PCR. About 38.3 % of the neonatal samples taken upon birth were found to contain herpesvirus genomes. When the DNA of VZV, HHV6 and HHV7 have been tested separately 7.6 % of the samples have been found to be positive strongly suggesting, that transplacental transmission of these viruses might occur (17, 132). Several other contradictory results have also been published (14, 55, 60, 84, 153, 178, 206). The problem with the methodology of the Australian authors was the complicated system of multiplex PCR assay they used. Cross reactions of different herpesviruses, and reduced sensitivity of the procedure due to the multiplex tests do not allow precise quantitative calculations (72).

EBV and HHV8 have been shown to be latently present in the B lymphocytes of the people (168, 187, 218). HHV6 and HHV7 have been shown to be latently harboured by T helper cells of about 50 to 70 per cent of adult populations (13, 114, 153, 179, 229). HCMV could be detected in the

CD34+ dendritic cells of people infected previously with the virus (167). The possibility is existing that among the cells translocated to the fetal tissue latently infected cells might be present carrying DNA of lypmphotropic herpesviruses in episomal form. Series of findings supported the latter possibility since presence of viral DNA or viruses have been detected in AF, urine or fetal tissues of healthy neonates (12, 14, 84). Previous publications have been reviewed by Pass RF recently (153).

1.4. Factors, which would be required for transplacental transmission of latent viruses

1.4.1. Structure of the placenta

The main layers of the tissues of placenta are shown in Fig. 1. (158). The muscles of the uterus are coverd by *decidua parietalis.* This layer continues below the placenta and it is called *decidua basalis.* The whole inner surface of the *myometrium* and *decidua* is covered by the *chorion* and *amnion* membranes of fetal origin. The placenta developes in between the decidua and amnion membranes. The outer surface of the cotyledons with the fetal blood vessels are covered by the chorionic plate. Their inner surface is covered by the syncytiotrophoblast layer (Fig. 2) separating the maternal and fetal tissues (140, 191). At the termini of the „villous tree" the villi provide sufficiently large syncytiotrophoblast surface to allow transport of oxygen and maternal substances into the fetal blood vessels in addtion to the removal of waste products of fetal metabolism. The section of the villi are shown in Fig. 3 (177).

Maternal blood is circulating in the intervillous space. The layer of syncytiotrophoblasts separate fetal mesenchymal tissue from maternal blood,

and fetal capillary endothel (FCE) separate fetal blood from the microvillous environment containing cytotrophoblasts, Hofbauer macrophages (HO) and fetal mesenchymal cells (177).

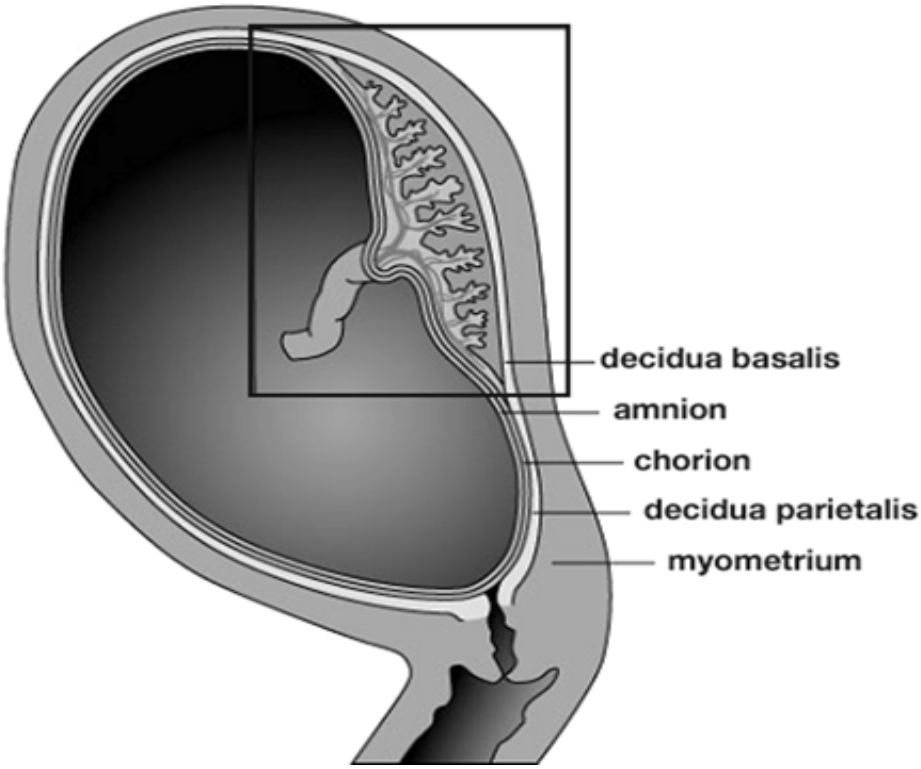

Figure 1. Schematic drawing of the human placenta showing the amnion (fetal part) decidua basalis and decidua parietalis (maternal part; Ref. 140)

1.4.2. Factors which might enable penetration of solutes or particles from the maternal blood into fetal capillaries of microvilli

Infection of fetal organs with genotype E of human immunodeficiency virus type 1 (HIV1) even in the 1st trimester of pregnancies has been shown recently (24). The authors concluded that chorioamnionitis, villitis, villous stromal fibrosis, placental infarction, deciduitis, plasmacellular deciduitis and

decidual necrosis might be responsible for virus transmission. Fetal glial cells, mesenchyme, alveolar and intestinal epithelium had been found to contain either p24 or proviral DNA. The difference between prenatal transmission and

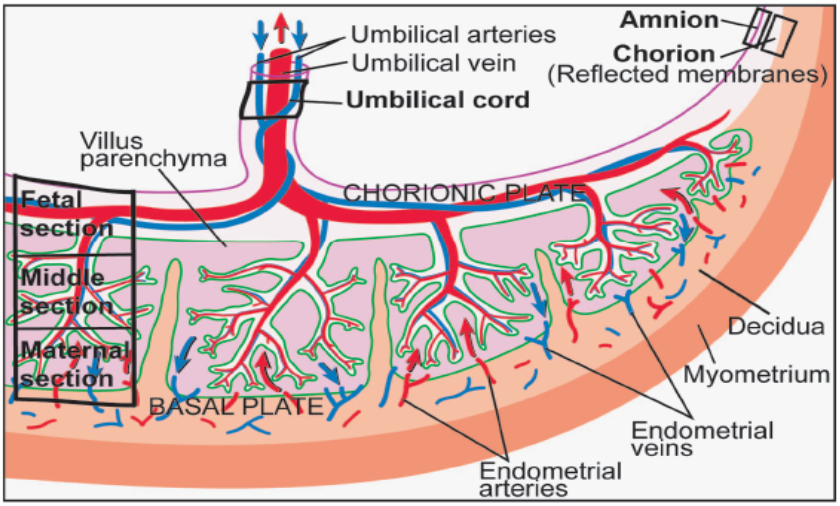

Figure 2. Schematic representation of a human term placenta. Umbilical arteries (blue colour) and vein (red colour in the umbilical cord). Chorionic plate composed of fused amnion and chorion membranes (Reflected membranes) villus parenchyma, basal plate and decidua supporting the endometrial arteries and veins of maternal origin (191).

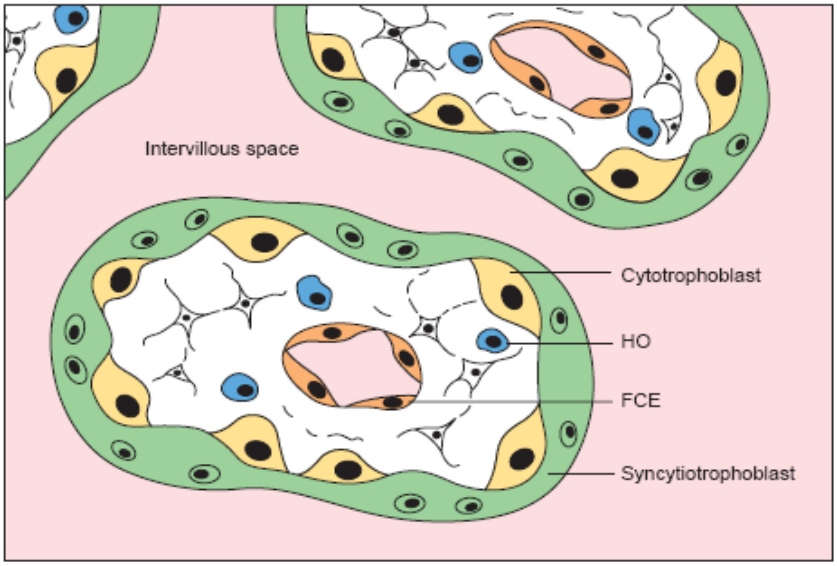

Figure 3. Schematic drawing of floating villi and the syncytiotrophoblast (green colour) separating maternal blood and fetal mesenchymal tissues (177). On the fetal side, cytotrophoblast cells (yellow colour), Hofbauer macrophages (HO) and fetal capillary endothelial cells (FCE).

and perinatal transmission has been found significant in chorioamnionitis, plasmacellular deciduitis and decidual necrosis.

It has been shown that HSV and EBV might cause deciduitis and villitis, thus creating the route of their own transplacental transmission (14).

Deciduitis, villitis and presence of HCMV in fetal endothelium has been described (96). Infectious mononucleosis of EBV origin resulted in perivasculitis, necrotising deciduitis, mild chorionitis and occasional vascular obliteration (145).

1.4.3. Specific adsorption and penetration of herpesviruses to susceptible cells

Herpesviruses possess a series of glycoproteins on their surface. These are labelled as gB, gC, gD, gE, gG, gH, gI etc. Heparin had been shown to inhibit adsorption of herpes simplex virus. The process of virus-cell interactions was shown to be a stepwise process with participation of viral and cellular glycoproteins ending with the fusion of the viral envelop and cellular membrane (Fig. 4).

The virus is first adsorbed by ionic forces to the chondroitin sulphate or other heparin-like mucopolysaccharides (21, 23) to glycoproteins of the cell membrane (green lines at the left end of Fig.4 (63, 140, 191). The first specific step of herpesvirus adsorption is the interaction of the viral glycoproteins with the specific host cell receptors. These are the epidermal growth factor receptor for HSV, CD21 for EBV, CD46 for HHV6 and CD98 for KSHV (see insert in Fig. 4.). These interaction (docking) is believed to be responsible for the different cell-specificities of herpesviruses.

The virus adsorbed to the receptor is accumulating integrins and interleukin receptors to the docking complex. These postattachment events activate innate immunity and induce the production of interleukins.

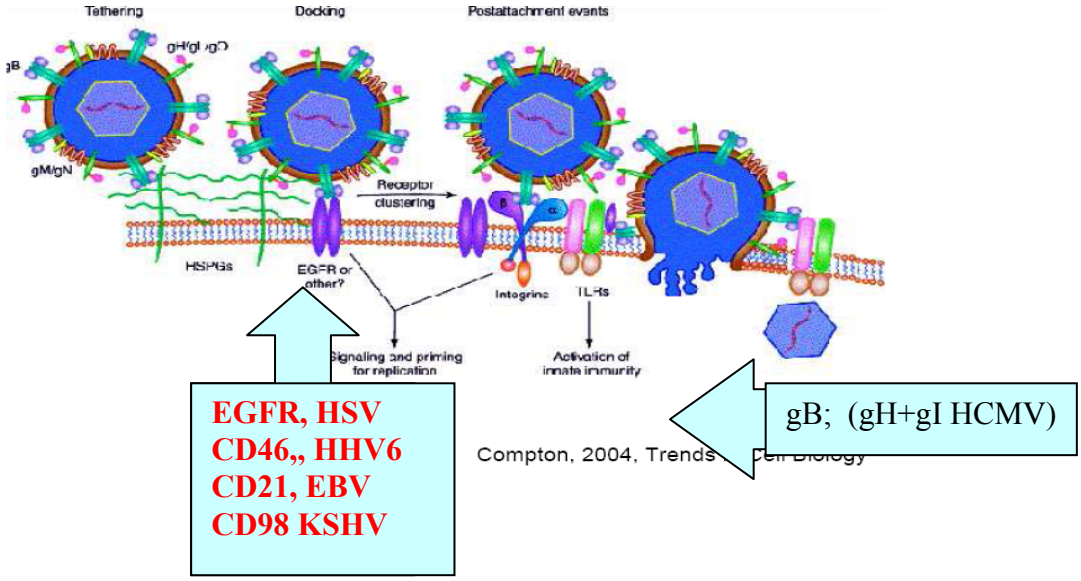

Figure 4. Adsorption and penetration of the herpesviruses into the host cells (63 and Compton: Trends in cell Biology). Green dimers on the viral surface represent gB (the largest) glycoprotein of herpesviruses. The green monomers with the red residue represent glycoproteins gH, gI and gD. Receptor clustering results in the accumulation of integrins and interleukin receptors (ILRs) to the virus carrier complex (postattachment) and the integrins and ILRs create signals, wich activate the cell and certain factors of the innate immunity (28, 92). The final step of the process is fusion of the viral envelop and cellular membrane (the blue tegument and the viral core particle enters the cytoplasm). The fusion has been shown to be initiated by the glycoproteins (shown in the light-blue arrow).

All gB proteins of herpesviruses tested, were found to carry a disintegrin-like amino acid sequence probably participating in this third postattachment complex (Fig. 5). The transmembrane signals initiated by this complex create the cellular environment for replication. The interleukin receptors activate innate immunity probably through toll-like receptors (28, 92). During this events the viral envelop and the cell membrane comes into close contact to each other, and the membrane fusion process will be induced

by viral glycoprotein gB in the case of EBV, HSV, HHV6 and KSHV (134). Both HHV6A and B genotypes have been able to cause cell to cell fusion "from without" (154). Fusion "from without" is the definition for membrane fusion when no virus replication is required for fusion and UV inactivated particles can induce cell-cell fusion, too. Uptake of HCMV by cell-cell fusion depending on pH has been also recognised and is associated probably with the wide range of cell types infected (176). Cell-cell fusion "from without" caused by HCMV glycoproteins (gH/gL) has been recognised recently (108).

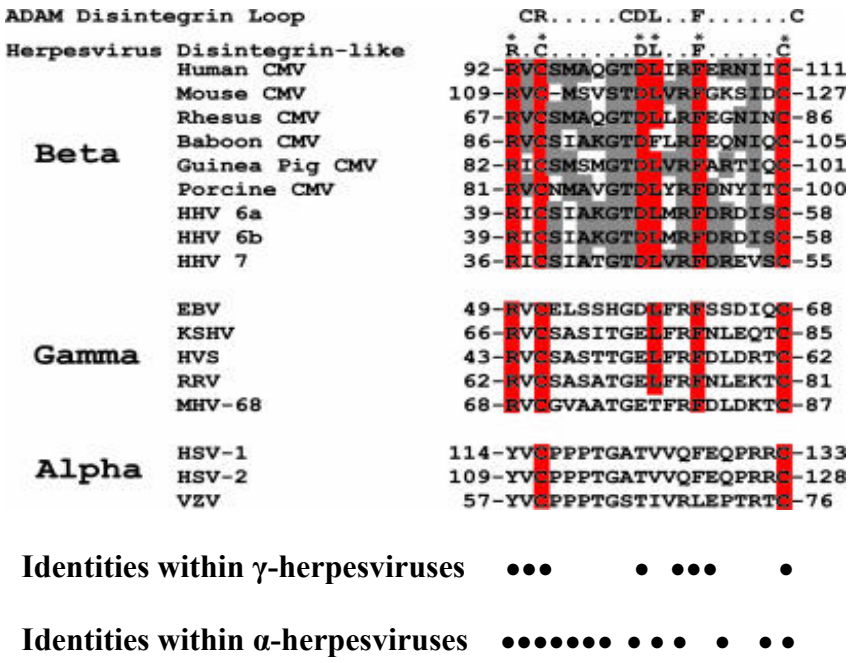

Figure 5. The comparative analysis of herpesviral gB amino acid sequences probably participating in the formation of a disintegrin loop (63). From the 20 residues shown 6 were found to be identical in β-herpesviruses (with one exception). Five of them were found to be identical in γ-herpesviruses, and only α-herpesviruses possessed 2 identities, probably because of the different host-range of these viruses. It has to be noted, that within the α group 13 of 20 residues were found to be identical (black dots below the amino acids). Within the gamma herpesvirus subgroup 8 of 20 amino acids are identical.

Disintegrins are called „ADAM" (A disintegrin and metalloprotease) transmembrane proteins. The ectodomains represent deadhesion and adhesion

part and a cystine rich fusion domain. The protease active site had not been conserved, therefore their role is probably adhesion or fusion (213). Their presence in the envelopes of herpesviruses might be involved in the fusion "from without" activities of herpesviruses.

1.4.4. In vitro *susceptibility of the syncytiotrophoblast cell to viruses*

A series of publication have revealed that syncytiotrophoblast cells can be infected *in vitro* with different viruses (15, 42, 64, 73, 114, 212). Double-infections with HIV-1 (73), or HTLV-I (211, 212) had been found to stimulate mutually the replication of EBV (211), CMV (Fisher et al. 2000; 212) or HHV6 (42) and HHV7 (73).

The presence of cytokines stimulates viral replication in syncytiotrophoblast cells (15, 16) *in vitro*. Thus, the simultaneous presence of endotoxin (LPS) and viruses in the blood of the mother may cause higher risk for the replication of viruses in the syncytiotrophoblast cells due to cytokines shown to be induced by endotoxins (LPS).

Once the cells of the feto-maternal barrier (syncytiotrophoblasts) have been infected by viruses and stimulated by cytokines, the spread of the virus(es) may only occur in the fetal tissues, when the receptors summarised in Figure 4. have already been differentiated in certain fetal organs.

1.4.5. Presence of specific virus receptors in fetal tissues

The presence of maternal hematopoetic cells carrying receptors for EBV (complement receptor CD21; 187) have been shown in fetal organs. Their presence among cord blood cells could be confirmed both in the 2nd and

3rd trimester (76, 136, 226). Fc-receptor dependent transcytosis of the host cells have been found to enhance or inhibit penetration of HCMV into placental cells (125). Expression of Fc-receptors has been shown in endothelial cells of the full-term placenta (138, 177). In mice teratogenic peptides could pass the placenta via immunoglobulin transporters (113). This mechanism might also promote or inhibit mother to fetus transmission of all lymphotropic herpesviruses.

The spread of herpesviruses within the fetal tissues might occur by cell-cell fusion "from without" shown to be caused even by the recombinant gB glycoprotein of EBV alone (134).

Receptor for HHV6 (CD46, a member of the complement system) has been shown to be present on fetal cells, too (112, 222). Preceding the nationwide vaccination programme with live measles vaccine, severe intrauterine complications caused by transplacental measles infections showed that the CD46 receptor had been present on fetal cells already in the first trimester of the fetal development. Cell-receptor complex of KSHV similar to other herpesviruses contains heparane sulfate (3) and the CD98/xCT transporter (102, 218). The cationic amino acid transporter has been found also in fetal cells being important in the fusion of syncytiotrophoblast cells (56, 116, 139). ICAM-I, a receptor for many human viruses, has been shown to be over expressed in trophoblast cells in the case of placentitis (101).

Perinatal and transplacental infection of HCMV, HHV6, HHV7 and EBV has been suggested previously (7, 11, 12, 32, 73). Differentiation of dendritic cells induced reactivation of HCMV from latently infected CD34+

granulocytic progenitor cells (167). In addition, HHV6 latency has been confirmed not only in T4 lymphocytes, but also in monocytes (114).

These findings support the possibility that maternal viruses may have contact to the syncytiotrophoblast. Their replication is possible in the syncytiotrophoblast cells and different cytokines, inflammation, villous necrosis and dual infection can facilitate their replication and penetration into the fetal mesenchyma.

Maternal cells also have been detected in fetal tissues (76) and feto-maternal virus transfer has been shown to be induced after cell to cell contact (118). The fetal-maternal transfer of *Toxoplasma gondii* is also mediated by ICAM-I adhesion of monocytes to trophoblast cells (2, 157). The finding that HHV6 has been able to be latently present in monocytes (114) suggest that monocyte-trophoblast contact may also be a mechanism of transmission, too. Erythrocytes infected with Plasmodia are in direct contact with infected cytotrophoblast cells, too (38).

The presence of known cell-receptors for lymphotropic herpesviruses have been detected in fetal tissues, but their appearance had not been followed precisely in association with the ontogenetic development of the fetus. It is known, however, that the immune response of fetal character begins between the 20^{th} - 22^{nd} weeks of pregnancy indicated by the appearance of IgM type fetal antobodies (26). Fetal B and T cell markers have to be present on fetal hematopoetic cells, too. The appearance of Fc-receptors (65) may support uptake of viruses or viral immune complexes into the fetal cells in the fetal environment.

Integrins have been also detected in fetal cells (58) as early as in the first trimester of the pregnancy. Different other immunomodulatory proteins have been found on the surface of macrophages in animal and human organs and in the placenta (52, 156, 165).

1.5. Other factors influencing maternal-fetal transmission of viruses

Endotoxins were shown to induce production of interleukins and growth factors in fetal tissues (123, 124). Cytokines have been also found in AF samples in connection with preterm labor and premature delivery in pregnant women, ruminants and mice (26, 39, 50, 87, 98, 197). The consequence of microorganisms and high endotoxin concentration has been shown to cause premature delivery, too (39, 60, 173, 174, 197). *In vitro* studies indicated that in amnion-derived cell lines IL-6 and IL-8 can be induced by a series of regulatory substances including endotoxins (62, 98, 104). It has been shown in *ex vivo* experiments that healthy placentae did not allow penetration of interleukins (1). However, in connection with intra-amniotic inflammation or maternal toxaemia or beginning eclampsia, the toxic alterations of microcapillary vessels of placenta were shown to facilitate penetration of cells and other substances, including endotoxin, into AF (95, 136, 227). Thus the presence of endotoxin in AF and dual viral infections of the pregnants might probably also facilitate the transplacental invasion of viruses.

1.6. Human papilloma viruses: a distinct family of perinatally and/or transplacentally transmitted viruses

Genital reactivation of HPV has been shown in pregnant women, but the viruses disappeared within 2 weeks following parturition (45, 221). The reactivation can be an explanation why papillomaviruses have infected babies during delivery (32, 135). High risk papillomaviruses have been also found to be transmitted vertically (33, 127, 135, 169,170, 183, 189). For example, transplacental transmission was evident in the case of a neonate born with developed condyloma acuminata (172).

According to the classical dogma, papillomaviruses are unable to spread in the form of viremia. Papillomaviruses only have been detected in white blood cells and in plasma samples of patients with invasive cervical carcinoma (180). Occurrence of transplacental transmission of papilloma viruses, however, similar to herpesviruses had been previously also suggested (9, 10, 172, 183).

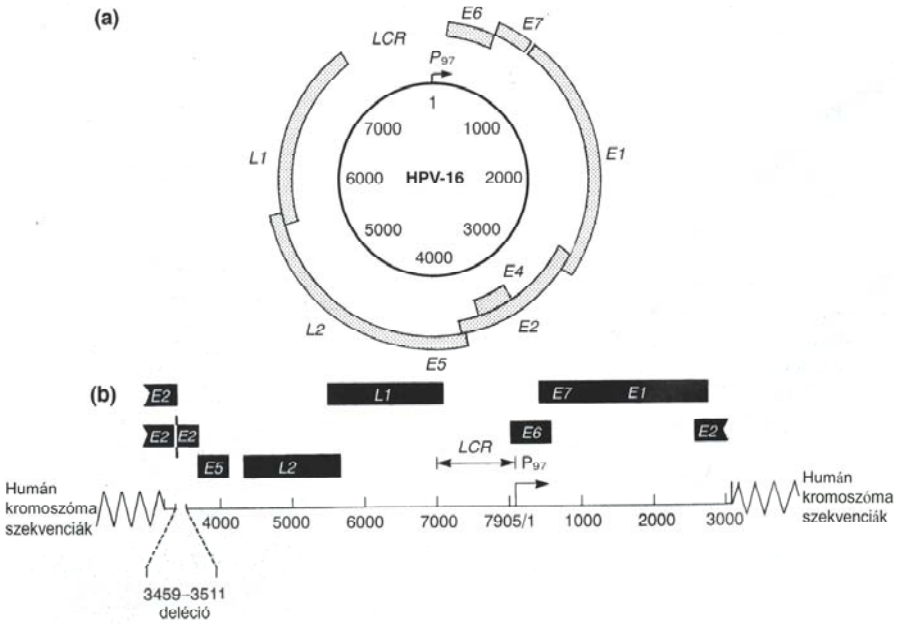

Figure 6. The simplified genetic structure of the extrachromosomal and integrated human papillomavirus genome (Czeglédy).
The grey boxes around the circular double-stranded genome (a) indicate the locations and reading frames of viral genes. The sequence corresponding to "L1" has been cloned and used as internal control (IC) for the

determination of copy number of viruses identified in the amniotic fluid. The verification of the genome types occurred by nucleotide sequencing of the same genomic region (100). The integrated genome is shown below (b). The dark rectangulars represent the locations of the late genes L1, L2 and early genes (E1 to E7) and their deletions in the cancer cells. *LCR* represents the Long Control Region of the genome.

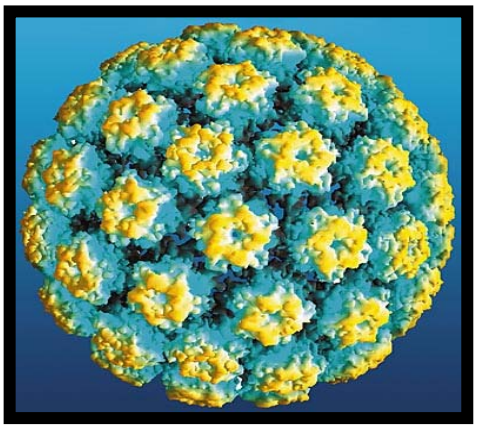

Figure 7. Computer generated image of the human papillomavirus particle. The yellow surface of the 72 pentamers carry the antigenic sites and the binding sites of the LN5 molecules

The problem of transplacental transmission arose again, when the EVER 1 and 2 genes had been discovered and suggested to be responsible for the generalisation of *epidermodysplasia veruciforme* (146). It is difficult to understand how viruses spread all over the surface of the body without viremia or transplacental transmission.

The receptor for papillomaviruses 11 and 40 have been found to be laminin 5 (LN5), a constituent of basal matrix, and not that of the cell membranes (43, 44). Heparane SO_4 contributed to the uptake of papillomaviruses by dendritic cells similar to herpesviruses (3, 29). LN5 was shown to be secreted after skin injuries by keratinocytes, and found to influence cell motility (215).

1.7. Definitions for different forms of vertical virus transmission

The detailed analysis of the literature suggested to differentiate and create definitions for the perinatal transmission and transplacental transmission as two different forms of vertical virus transmission. Some viruses (endogeneous retroviruses transmitted through the germe line) have to be excluded from these definitions.

1.7.1. Perinatal virus transmission:

When the source of infections are genital secretions, maternal blood contaminations or herpetic erruptions of the mother during delivery, the penetration occurs through injuries, mucous membranes, or exposure of the surface and openings of the neonate;

1.7.2. Transplacental virus transmission:

When the maternal-fetal barrier is penetrated by microorganisms mediated by infection of the syncytiotrophoblast cells through cell to cell contact of maternal macrophages or contact with other maternal cells and cytotrophoblasts; when Fc-receptor mediated transfer of immunocomplexes containing viruses occur; when transfer of maternal hematopoetic cells carrying latent viruses facilitated by placentitis, villitis, placental necrosis, cytokines and/or endotoxins before the onset of delivery takes place. The vertical transmission of viruses to the fetus might be mediated by the amniotic fluid, when the replication of viruses occurs in the cells of fetal placenta.

The examination of amniotic fluid provides an excellent possibility to clarify some aspects of transplacental transmission of viruses and its endotoxin facilitation.

2.) Objectives

The work is composed of four major parts.

2.1. Seroepidemiological examination of patients suffering from various syndroms of hepattitis B virus (HBV), hepatitis A virus (HAV) and hepatitis C virus (HCV) infections in the Southern Plain Region of Hungary.

Comparison of the findings with the results of the seroepidemiological survey performed in 1999-2000 representative for the whole country. The results may serve as basis for further seroepidemiological surveys following introduction of the mandatory nation-wide vaccination of the school-children at the end of their studies (14 to 15 years of age) launched in 2001 in Hungary.

2.2. The subtyping of hepatitis B viruses within this region using DNA sequencing.

2.3. The comparison of the HBx-specific serological markers in symptomless carriers from this region with that of the patients suffering from acute HBV hepatitis and chronic hepatitis using the serological procedure developed by Pál J et al. (148, 149).

2.4. Recent results suggested that the transplacental transmission of hepatitis B virus, and HIV is possible, thus it has been decided to carry out systematic examination of amniotic fluids for the presence of the DNA of lymphotropic herpesviruses and papillomaviruses in order to provide evidence that transplacental transmission of other viruses may also occur at the end of healthy pregnancy. Endotoxin was also examined as a possible facilitating factor of transplacental transmission. This type of examination could not be extended to symptomless hepatitis B carrier pregnants, since only 0.4 % of

the pregnants are symptomless carriers and the sample collection in acceptable number could have been very difficult. The safety measures in connection with the deliveries of HBV carrier mothers would also hinder careful sampling of amniotic fluids.

3.) Materials and Methods

3.1) Serum samples.

Serum samples for the serological survey were collected in the Csongrád county. About 30,000 serum samples were collected from 17,994 persons between 1993 and 2001. The samples were tested for the presence of serological markers of classical hepatitis virus markers. The parents did not agree with sampling of their children, therefore only 69 serum samples were obtained from these children and teenagers below 19 years of age.

Frozen residua of serum samples taken for diagnostic purposes were stored in the frozen state for the examination of anti-HBx antibodies, and HBxAg surveillance. The samples of 12 symptomless chronic carrier pregnant women were selected from those analysed before (144). Sera of 14 acute HBV-patients were selected from the diagnostic material of the Hepatitis and Molecular Diagnostic Department of the National Center for Epidemiology.. Remainders of sera of 80 chronic hepatitis patients were also used, who were systematically retested time to time for the presence of HBV-DNA. The negative controls were taken from 22 healthy blood donors (148, 149).

Maternal blood samples were taken at the National medical center, Budapest immediately upon delivery and the tubes with vacuum-safe caps were fixed to the tube with the corresponding amniotic fluid sample using

adhesive tape. Whole blood samples of the the women were frozen without fractionation in order to make possible later detection of antibodies and viral nucleic acids present in both the plasma and within the nuclei of white blood cells.

3.*2) Amniotic fluid samples*

The Institutional Review Board of the National Medical Center, Budapest did not approve collection of trans-abdominal amniotic fluid (AF) before Caesarian sections. Thus a special trans-vaginal sampling of AF had to be planned in order to prevent contamination of the samples with cervical or vaginal secretions, which might contain HCMV (228), EBV (59), HHV2 (119), HHV6, HHV7, HHV8 (31) and papillomaviruses (89).

The Model 21 Gx1 (#450072) needle has been mounted to the multi-use holder (#450201, Greiner and Co., Austria) and the rupture of amniotic membrane was performed under gynecological exposure of the orifice uteri similar to that applied for colposcopy. The vacuette syringe was applied to the inner end of the needle completely covered by a rubber layer within the multi-use holder. The rubber layer was only perforated when the vacuum-tube was attached to it. Tubes and the Multi-Use holders were conform to 21CFR 862.1675 Blood specimen collection device (Vacuette, Greiner and Co., Austria). The system possessed also FDA licence granted according to the US requirements.

When the lower end of the needle perforated the rubber cover of the vacuette tube the vacuum has sucked the amniotic fluid. Afterwords the tube was detached from the system. The outer surface of the tube could be contaminated by virus-containing secretions, but the inner content had never

been in contact with the outer surface. The tubes were placed immediately to -20 ^{o}C and transported in frozen state together with the frozen whole blood sample of the same woman to the laboratory. The tubes were fixed to each other using adhesive tape. In case of outer contamination, both tubes (i.e. amniotic fluid and blood) should be positive for the same virus. No such result has been obtained.

In the laboratory the tubes were melted at room temperature, vortexed to obtain homogeneity of the samples and centrifuged at 3000 rpm for 10 minutes at + 4 ^{o}C before opening their airtight cap. The airtight caps were immediately discarded upon removal one by one, and the supernate and sediment containing meconium and cell debris were pipetted using disposable tips into separate, new sterile tubes with screw caps.The original tubes and caps were subsequently autoclaved and destroyed.

One hundred-six AF samples were collected. The amounts of AF and maternal blood (B) samples ranged between 4 to 10 ml.

3.3 Serological techniques

HBsAg tests were undertaken together with other prenatal diagnostic screening using 2nd generation test kits HBsAg Uniform II manufactured by Organon Teknika (The Netherlands). All samples found positive for HBsAg were retested using the Confirmatory reagent kit of Organon Teknika.

Anti-HAV IgG and IgM reagent kits manufactured by Organon Teknika were used. Reagent kits purchased from Sorin Biomedica (Italy) were used for the detection of anti-HCV (mixed antibodies).

The reagents were used according to the protocols of the manufacturers. The O.D. values were measured in Micro-ELISA Reader Type 530 of Organon Teknika.

The ELISA technique for the quantitative detection of HBxAg was developed and performed in Pécs by Joseph Pál (148, 149). Briefly the microplates were covered by the monoclonal antibody of clone # 3F6/G10 specific to HBxAg (carbonate buffer pH 9.6). The wells were washed three times with PBS containing 0.2 % Tween-20. The protein-free surface of the wells was covered by 0.5 % gelatine (Sigma, USA). 12 serial dilutions of 1:2 steps were prepared from the recombinant antigen (HBxAg-GST = Glutathion S-transferase fusion protein with HBxAg) using 1 to 10 dilution of normal human serum. Before addition to the wells, the dilutions were incubated in glycine buffer (pH 2) for 10 min at 56 ºC, than adjusted to pH 8-9 with 1 M Tris-HCl. The samples to be tested were also treated in 1 to 10 dilution similar to the recombinant antigen dilutions. 100 µL of serum dilution and 100 µL of serial dilution samples of standard HBxAg-GST were pipetted into the wells. Following 6 hours incubation at 37 ºC the unadsorbed material was washed trice, and the detecting second monoclonal antibody (clone # 4F1/A9) conjugated by streptavidin-peroxidase was added and incubated for an additonal 1 hr at 37 ºC. The reaction was visualised using o-phenylene-diamin (OPD) and measured at 492 nm. In order to standardise the O.D. 492 values, the GST-standard curve was applied.

Indirect ELISA for the detection of HBxAg specific human antibodies: The microplates were coated with HBxAG-GST fusion protein solution. All sera were pretested with GST in order to exclude aspecific reactions. The

specific antibodies were examined in serum samples diluted 1 to 100. The visualisation of the results was performed using horse raddish peroxidase (HRP)-labelled IgG- or IgM-specific monoclonal antibodies (DAKO, Denmark). The results were visualised by OPD as above.

HHV6-specific antibodies in the blood were detected using HHV6 IgG EIA kit (Biotrin International Ltd, Dublin, Cat. No.:V3HHV6).

3.4. Detection of endotoxin

Endotoxin tests were carried out at first from the samples in order to prevent any external contamination. 500 µL of 50 supernates was pipetted into endotoxin-free Eppendorf vials using atoxic pipette tips and the LAL test was performed with duplicate samples according to the protocol of the manufacturer (*in vitro* semiquantitative reagent Limulus Amebocyte Lysate Pyrotell® form *Lymulus polyphemus;* 73, 74). Residual blood contamination and meconium was shown previously by others not to interfere with the sensitivity of LAL tests (73). Results were expressed in endotoxin units/ml (EU/ml). Dilutions of standard endotoxin reagents were used for the calculation of semiquantitative results. The LAL tests have been performed in an accredited laboratory (Medyag, Budapest) specialised for the quality control of therapeutical biological products.

3.5. Molecular detection of viral nucleic acids

3.5.1. Detection of DNA of herpesviruses using nested PCR

Supernates and sediments of AF samples and blood samples (160 µl each**)** were mixed with lysis buffer (Tris-HCl 0,2 M, pH 7.5, 25 mM EDTA, 0,3 M NaCl, 2% SDS and Proteinase K, SIGMA Co.) and incubated for 1.5

hour at 50°C in water bath. Deproteinisation was done using Tris-buffered phenol (SIGMA Co.) and chloroform (Reanal, Budapest) at ambient temperature. The aqueous phase was supplemented with 0.2 M Na-acetate and precipitated with ethanol at -20 °C overnight.

DNA precipitates were washed twice with 70 % precooled ethanol (Eppendorf microfuge) and dried for 1 hour at room temperature. The DNA was redissolved in 20 µL nuclease free distilled water and stored at -20 °C until PCR amplification. Nested PCR was done with primers specific for DNA polymerase genes of lymphotropic herpesviruses (162), but in contrast to the reference no "multiple PCR" was applied in order to make the technique as sensitive as possible.

HHV6 antibodies in the blood were detected using HHV6 IgG EIA kit (Biotrin International Ltd, Dublin, Cat. No.:V3HHV6).

3.5.2. Cells and viruses used as positive controls

EBV DNA was prepared from B95-8 cells (ATCC: CRL 1612). AD-169 (ATCC: VR-538) strain of HCMV was grown on primary human fibroblast cells. HHV6 DNA was prepared from commercial immunofluorescence slides purchased from Biotrin (Dublin, Cat. No.:V3HHV6). Spots of infected cells were suspended in lysis buffer (25 µl/spot) for 10 min at 37 ^{o}C and collected for DNA isolation. BCBL-1 cells (producing HHV8) were grown in RPMI-1640 completed with 2-mercapto-ethanol (5µM) and used for DNA isolation (168). Verification of HHV6 and HHV7 amplimers was done by sequencing (described later in paragraph 3.5.4). Following verification „in house" DNA-positive HHV7 serum was

used as positive control. Echovirus 11 suspension was used for the detection of 5'-NTR of enteroviruses (57, 99, 198) for aspecific detection of potential contamination with vaginal secretion.

The sensitivity of the nested PCR techniques were between 200 and 300 copy number/ml calculated on the basis of the results obtained with the control panel of an international proficiency test for HCMV and EBV.

3.5.3. Detection and typing of papillomaviruses in the amniotic fluid

Anogenital HPV genotypes subgenomic fragments (of genotypes 6, 11, 16, 18, 31, 33, 35, 39, 42, 45, 51, 52, 55/44, 58, 59, 66, 68) containing the *L1* gene were cloned by the firm GenoID, Budapest into the pCR2.1 Topo vector (Invitrogen, Carlsbad, CA) to serve as internal controls (100).

Total DNA was prepared from the high speed sediments of supernates of 96 AF in duplicate. HPV-specific recombinant internal control (IC) DNA was added and processed together with the sample DNA. PCR was performed with L1F and L2R primer sets (100) amplifying 46 anogenital HPV types. The probes (Integrated DNA Technologies, Coralville, IA) used for solid-phase hybridization were fluorescein-labelled in case of genus and genotype specific oligonucleotides, and digitoxigenine-labelled in case of "HPV-Internal-Control-specific" (HPV IC-specific) oligonucleotides. All labelled nucleotides were HPLC purified. Results were considered to be unequivocal if the IC product was present visualized by agarose gel electrophoresis.

The biotinilated PCR products (products of amplification by one labelled primer) were captured onto the surface of a streptavidin-coated microplate well. The complementary chain was eluted from the immobilized

PCR product and the hybridization was carried out in the presence of specific fluorescein- (in case of genus and genotype specific probes) and digitoxigenin- (in case of IC-specific) labelled probes. The bound probes were then reacted with anti-fluorescein-HRPO (horse-radish peroxidase) and anti-DIG-AP (AP-alkaline phosphatase) antibody. Substrate development was done separately. Fluorescence signals of HRPO substrate was measured at ex/em: 324/410 in Microfluor®2 (Thermo Electron Corporation) while the substrate of AP yielded fluorescein signals measured at ex/em: 355/460.

3.5.4. Nucleotide sequencing of HPV and HBV, HHV7 and HHV8 PCR products

The PCR products were obtained by one of the procedures decribed under paragraphs 3.5.1, 3.5.2. or 3.5.3. Sequences marked on the genome of human papillomavirus genome shown in Figure 6. The regions of hepatitis B virus were amplified or cloned for sequencing as shown in Figure 8. PCR products (196) were purified by High Pure PCR Product Purification Kit (Roche Diagnostics GmbH, Mannheim, Germany), sequenced using BigDye Terminator Cycle Sequencing Kit and run on ABI Prism 300 Genetic Analyser (Applied Biosystems). The DNA sequences were aligned using the BLAST algorithm.

To assess the specificity and sensitivity of the nucleic acid amplification method for detection of HBV, a proficiency panel designed by the Quality Control for Molecular Diagnostics was tested. The sensitivity of the PCR method was less than 200 copies/ml according to the proficiency panel of QCMD. For direct sequencing, PCR products

were purified using a PCR Clean up-M Kit (Viogene). An ABI PRISM 3.1 BigDye Terminator Kit (Perkin Elmer) was used according to the instructions of the manufacturer. Electrophoresis was carried out on an ABI PRISM 3100 Genetic Analyzer.

Table 1. PCR primers used to amplify a segment of the surface protein coding region of HBV

Name	Sequence (5'–3')	nt position
Outer1	TCA CCA TAT TCT TGG GAA CAA GA	2823–2845
Outer2	CGA ACC ACT GAA CAA ATG GC	704–685
Inner1	AAT CCA GAT TGG GAC TTC AAC C	2965–2986
Inner2	GAG GAC AAA CGG GCA ACA TAC	479–459

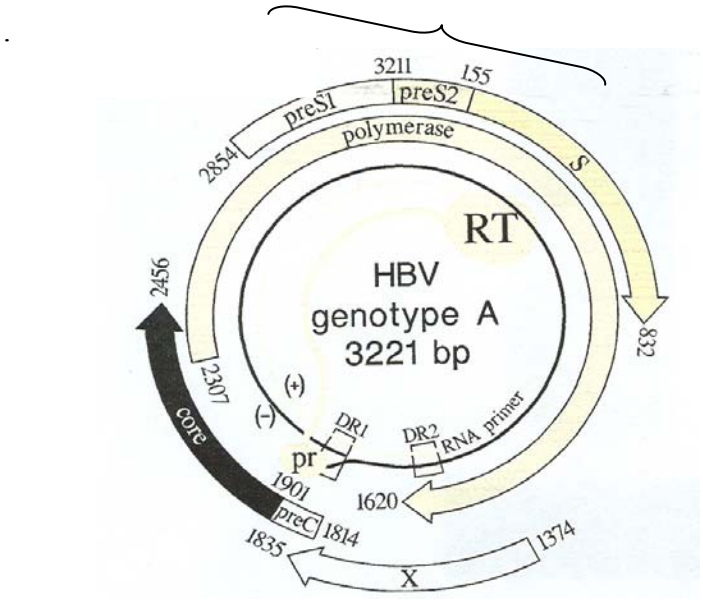

Figure 8. The segments of hepatitis B viral DNA examined in the frames of this work. The 143 amino-acid region of the *"X"* gene product has been cloned and expressed (128) and the X-GST fusion protein has been used for the serological testing of HBsAg positive sera (148, 149).

The subtyping, and sequence determination was performed on a region of "preS1-preS2-S" (arrow) of the genome between the nucleotides 2965 to 459 indicated by the arch. The location of the outer and inner primers are

shown in Table I. (202). RT is indicating the presence of reverse transcriptase in the particles. The other labels indicating different signals and sequences of the genome are not relevant from the point of view of this work.

3.6. *Statistical methods and evaluation*

For the calculation of significance Fisher's exact test and the Pearson Chi test run under Microsoft Windows were used.

4. Results

4.1. HBsAg carriers and other viral markers in the age groups tested between 1993 and 2001 in the Southern Plain Region

The ratio of HBsAg positive persons were found to be 82.5 % in the age group between 20 to 39 years of the hepatitis patients (Table II). Unfortunately the anti-HBcAg reagents could not be purchased for the testing of this group of patients. Therefore one could not assess the proportion of people recovered from HBV infection previously.

Only the 583 persons could be identified on the basis of the HBsAg, anti-HAV-IgM and anti-HCV positivity. HBsAg carriers were symptomless carriers of the virus or were suffering from acute or chronic hepatitis B infection. Sixhundred and ten of the 1193 persons were positive only for anti-HAV IgG (Not shown in Table II) indicating, that they recovered from epidemic hepatitis "A" earlier.

Double and triple infections with hepatitis viruses were also found among the patients. Anti-HAV IgM indicated acute HAV infection, the presence of HBsAg indicated acute or persistent HBV infection. Hepatitis C seropositivity was always considered to be the indicator of HCV syndrome. Multiple infections were the following: HAV+HCV - 5 patients, HAV + HBV

– 4 patients and HBV + HCV – 6 patients. Triple infections were detected in 3 patients.

Table II. Age distribution of patients with markers indicating acute or active viral hepatitis on the basis of the examination of 1193 patients of clinical diagnoses of hepatitis (184).

Age groups	0 - 19	20 – 39	40 – 59	> 60	Total
No of persons	69	189	452	483	1193
No. of hepatitis markers *	63	248 *	149	123	583
HBsAg	36	156	45	26	263
% + persons	57.1	82.5	10.0	5.4	22.0
Anti-HAV IgM	22	34	12	8	76
% + persons	34.9	18.0	2.7	1.7	5.5
Anti-HCV	5	58	92	89	244
% + persons	7.9	30.7	20.4	18.4	20.6
Data of general population for comparison taken from Ref. 131					
%anti-HBcAg pos.	1.9	5.47	12.16	14.94	N.T.
% HBsAg pos.	0.066	0.54	1.38	0	N.T.
% HCV pos.	0.18	0.45	1.3	1.2	N.T.

* Number of hepatitis markers indicating acute or persistant infection.

Table III. The proportion of viral hepatitis markers indicating acute or persisting hepatitis in different age groups of the low prevalence population tested.

Age groups	0-19	20-39	40-59	> 60	Total
No. of markers	63	248	149	123	583
HBsAg	36	156	45	26	263
% positive	57.1	62.9	30.2	21.1	45.1
Anti-HAV-IgM	22	34	12	8	76
% positive	34.9	13.7	8.1	6.3	13.0
Anti HCV	5	58	92	89	244
% positive	7.9	23.4	61.7	72.4	41.9

In Table I the per cent of patients with hepatitis markers are summarised. There are many of them, who had hepatitis syndrom, but the only virus marker was anti-HAV IgG. These people had probably hepatits because of other etiological factors (autoimmune, alcohol, biliary cirrhosis, EBV, CMV or viruses, which could not be not be tested, hepatitis E virus, or TTV). In Table two only the markers are plotted which indicate active virus multiplication. Double and triple infections were found to be only present in the age group from 20 to 39 years. The reason for that finding is, that the seropositivity of these people to HAV is only 20 % in contrast to the older groups with 60 and 80 %. A second explanation is the more intensive activities of the people between 20 to 39 years.

4.2. HBxAg and antibodies in sera of chronic symptomless HBV carriers and of patients suffering from acute and chronic hepatitis

A proportion of hepatitis B positive persons in all three groups possessed HBxAg in their sera of different concentrations (Figure 9). In each group, however there were individuals with HBxAg concentrations below the limit of detection. The standard curves and the control dilutions were published by J. Pál (148, 149) including the automatic calculation of O.D. values and cutoff.

The studies on the relative concentrations of X-protein specific antibodies, revealed that IgM-isotype antibodies were present even in the sera of symptomless carriers (Figure 10). Similarly all persons tested possessed IgG-isotype antibodies above the level of detection (above cutoff). The absence of detectable X-protien in the sera of chronic hepatitis patients is probably the result of the formation of immunocomplexes.

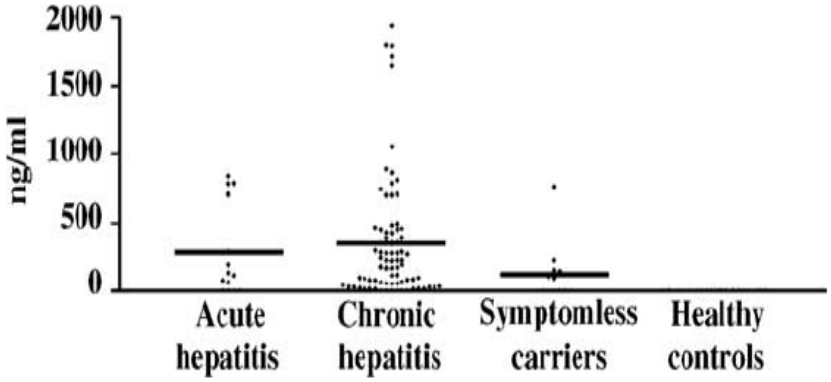

Figure 9. **Hepatitis B X-antigen concentration in the sera of patients suffering from acute, chronic and symptomless carrier infections. (148).** Number of acute HBV-patients was 14; no. of patients suffering from chronic B hepatitis was 80; No. of symptomless carriers was 12, control blood donors were also examined (22). The numbers indicate nanogram/ml concentrations of HBxAg obtained by sandwich-type ELISA technique. Horizontal lines indicate the mean of the X-antigen concentration.

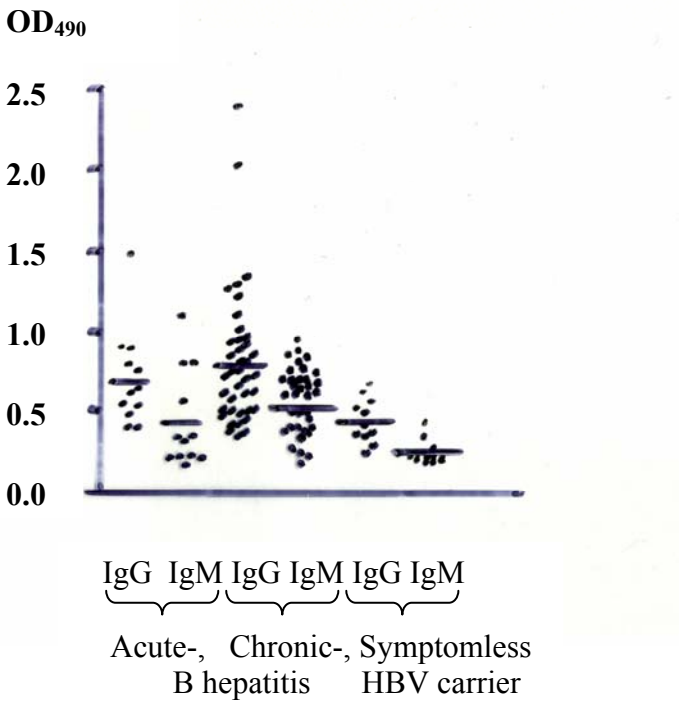

Figure 10. HBxAg-specific IgG and IgM type antibodies in the sera of acute- and chronic hepatitis B syndroms and symptomless HBV carriers. Each dot represents O.D. $_{490}$ values obtaind by the indirect ELISA technique.

4.3. The genotypes of HBV in the Southern Plain region of Hungary

Genotypes of the hepatitis B viruses were classified by nucleotide sequencing, although other methods are also available (86). Sequencing results classified all Hungarian viruses into genotypes "A" and "D". The viruses could be classified into different clusters of both genotypes even within the small Southern Plain (new administrative) region of the country (202). The geographical locations of "A" and "D" genotypes are shown in Figure 11.

Figure 11. Geographical distribution of HBV genotypes in the Southern Plain Region of Hungary. Circles indicate the origin of genotype "D", **triangles** show the source of genotype "A" samples. Both "A" and "D" HBV genotypes are present in this region, too (202).

Recombinant genotype was not detected among the samples examined from the Southern Plain Region of Hungary yet.

4.4. DNA of lymphotropic herpesviruses in amniotic fluid samples

In the majority of the AF samples the herpesviruses could be detected either in the "low-speed" supernate containing floating cells only or in the

sediment containing meconium and aggregated cells. As shown in Table IV. herpesvirus DNA was found in 26 of 106 AF samples. The respective number of HCMV, HHV7, HHV8, EBV and HHV6 was 9, 8, 5, 4 and 1. From 104 maternal sera 12 contained DNA of herpesviruses. The number of HHV8, HHV6, EBV, HCMV and HHV7 DNA positive sera was 7, 3, 1, 1 and 1, respectively (Tables IV and V).

The distribution of herpesviruses detectable in the supernates or cellular sediments of amniotic fluids are summarised in Table IV. EBV was present in detectable amount only in one supernate, but 3 cellular sediments were positive. The only HHV6 and all HHV7 viruses were found in the cellular sediments of AF. HHV8 DNA, however, was detectable exclusively in the supernates of AF. The HCMV was present in three samples simultaneously in the supernates and cellular sediments of AF. HCMV was detectable in one sample only in the supernate and in 5 samples exclusively in the cellular sediments. All HPV viruses (shown in Table IV and VI) were found in the supernates of the AF samples. The ratio of distribution of viruses in the blood and in the amniotic fluid was found to be unequal. The majority of HCMV, HHV7 and EBV was present in the AF (21 viruses in AF in contrast to 3 in blood). The prevalence of HHV6 and HHV8, however, was higher in blood than in AF (10 viruses in the blood in contrast to 6 in AF). This difference was found to be highly significant according to the Pearson Chi-square test ($p = < 0.001$).

Two women of 104 were found to carry viruses in both AF and blood. However, the viruses detected in the blood and AF of the same woman were

different excluding possibility of contamination (Patients #28 and #97 in Table IV).

Of 26 DNA-positive amniotic fluid samples 8 were found to contain DNA of more than one viruses (Table IV).

4.5 Relationship of viruses and endotoxin content of AF samples

The herpes- and papillomaviruses detected in amniotic fluid samples with and without detectable endotoxin concentration are summarised in Table VI. Fifteen amniotic fluids of 50 were found to contain measurable endotoxin concentrations. The range was from 0.03 EU/ml to 3.0 EU/ml in 15 amniotic fluids, but the endotoxin concentration was below the limit of detection in 35 samples (< 0.03 EU/ml).

Four of the endotoxin positive samples contained viruses. Two of the samples were found to be double infected (HPV types 18 and 45 in samples #A4 and #A41; in addition to a low risk HPV in sample #A4, HCMV in sample #A46; and 2 HHV8 in samples #A41 and #A43). Two of the 15 blood

Table IV. Herpesvirus and papillomavirus DNA in the amniotic fluids (26) and blood samples (12) of healthy mothers after normal parturition.

Asterisks show, that the samples were not tested for the presence of papillomaviruses. Only samples (106 amniotic fluids and 104 blood samples) positive for any of the viruses are included. ** Sample No. 63 without HHV6-specific IgG was found to be positive for DNA in the amniotic fluid. Blood sample No. 97 was found to be positive for HHV6 DNA in the blood, but HHV6-specific IgG was absent. This patient possibly has acquired an infection at the end of the pregnancy. The AF samples contained the viral DNA in some cases only in the supernate. In either cases the virus could be detected only in the cellular sediment of the samples. The later results were labelled by low-case “c” after the specification of the virus. When the detected virus was present both in the supernate and sediment, the latter is labelled as: “+c”

Serial No. of sample	EBV	HCMV	HHV6	HHV7	HHV8 KSHV	HHV6 IgG	HPV type	No. of +
4 A	Ø	Ø	Ø	Ø	Ø	Neg	18+LR	2
15 Blood	Ø	Ø	Ø	Ø	HHV8	+	N.D.	1
16 Blood	Ø	HCMV	Ø	Ø	Ø	+	N.D.	1
20 Blood	Ø	Ø	Ø	Ø	HHV8	+	N.D.	1
21 A	Ø	Ø	Ø	HHV7c	Ø	+	Ø	1
24 A	Ø	Ø	Ø	HHV7c	Ø	+	Ø	1
28 A	Ø	Ø	Ø	Ø	Ø	N.D.	Untyped	1
28 Blood	EBV	Ø	Ø	Ø	Ø	+	N.D.	1
29 A	Ø	Ø	Ø	Ø	HHV8	+	Ø	1
35 Blood	Ø	Ø	HHV6	Ø	Ø	+	N.D.	1
37 A	EBV	Ø	Ø	Ø	Ø	+	Ø	1
41 A	Ø	Ø	Ø	Ø	HHV8	Neg	45	2
42 A	Ø	Ø	Ø	Ø	HHV8	Neg	Ø	1
43 A	Ø	Ø	Ø	Ø	HHV8	+	Ø	1
46 A	Ø	HCMV	Ø	Ø	Ø	+	Ø	1
48 A	Ø	Ø	Ø	Ø	HHV8	+	Ø	1
50 Blood	Ø	Ø	Ø	Ø	HHV8	+	N.D.	1
53 Blood	Ø	Ø	Ø	HHV7	HHV8	+	N.D.	2
57 A	Ø	HCMVc	Ø	Ø	Ø	+	Ø	1
58 Blood	Ø	Ø	Ø	Ø	HHV8	+	N.D.	1
59 Blood	Ø	Ø	Ø	Ø	HHV8	+	N.D.	1
61 A	Ø	Ø	Ø	HHV7c	Ø	+	Ø	1
63 A	EBVc	HCMVc	HHV6c	Ø	Ø	Neg**	39+58	5
65 Blood	Ø	Ø	Ø	Ø	HHV8	+	N.D.	1
66 A	Ø	Ø	Ø	HHV7c	Ø	+	Ø	1
68 A	EBVc	Ø	Ø	Ø	Ø	+	18	2
76 A	Ø	HCMVc	Ø	Ø	Ø	Neg	62	2
78 A	Ø	HCMVc	Ø	HHV7c	Ø	+	Ø	2
91 A	Ø	Ø	Ø	Ø	Ø	Neg	58	1
92 A	Ø	HCMV+c	Ø	Ø	Ø	+	Ø	1
96 A	Ø	HCMV c	Ø	Ø	Ø	Neg	Ø	1
97 A	Ø	HCMV+c	Ø	Ø	Ø	N.D.	Ø	1
97 Blood	Ø	Ø	HHV6	Ø	Ø	Neg**	N.D.	1
100 A	Ø	Ø	Ø	HHV7c	Ø	+	N.D.	1*
101 Blood	Ø	Ø	HHV6	Ø	Ø	+	N.D.	1*
102 A	Ø	Ø	Ø	HHV7c	Ø	+	N.D.	1*
105 A	Ø	HCMV+c	Ø	Ø	Ø	Neg	N.D.	1*
108 A	EBVc	Ø	Ø	HHV7c	Ø	N.D.	N.D.	2*
Total (Viruses in amniotic fluids + viruses in blood samples)								
26 + 12	4 + 1	9 + 1	1 + 3	8 + 1	5 + 7	ELISA	7 of 96	

samples taken from mothers with endotoxin positive AFs contained HHV8 (#B15 and #B50). No one of the viruses occurred either in the blood or in the AF of the same mother (Table VI).

The number of viruses detected in these amniotic fluids was 4 (EBV in sample #A37, 2 HHV8 in samples #A42 and #A48 and one non-typable HPV in sample #A28 also shown in Table VI.). In the blood of mothers with endotoxin negative AFs EBV (#B28), HCMV (#B16), HHV6 (#B35), HHV7+8 (#B53) and 2 HHV8s (#B58 and #B59) DNAs could be identified..

These differences, however, were not found to be significant ($p = > 0.05$).

Antibodies to HHV6 were detected in the blood of 74 (70 %) women. Two mothers had no detectable antibodies in the blood (Table VI), but HHV6 DNA was present in AF of one of them (#A63) and in blood of the other (#B97).

4.6. Papillomaviruses in amniotic fluid samples

Papillomaviruses were tested in AF only. Seven of 96 samples contained HPV types 18, 39, 45, 58, 62 and a low risk virus (LR) in addition to a „non-typable" one (Tables IV. and VI). Retrograde contamination was excluded by testing the presence of herpes simplex type 2 in all 96 AF samples, but it was not detected by nested PCR in any of the samples (100). Universal (5'-NTR) enterovirus nested PCR was also performed with all samples positive for endotoxins (43), but their absence indicated also, that no contamination occurred (experiments not presented).

The sensitivity of the technique is shown in Table VII. Internal control recombinants (IC) revealed that the detectable copy number of HPV types was between 70 and 305 genome equivalents per sample. The range of sensitivity of the tests was shown to be between 29 and 1160 copies per aliquot. Table VI. shows also genogroups of HPV types and references in bracketts describing genotypes identified in Hungarian patients (45, 67, 115, 126, 199, 200, 220 in the right column of Table VII.).

Table V. Physical distribution of viruses in the supernates, cellular sediments of AF and blood samples.
The viruses could be detected either in the supernates or in the cellular sediments of amniotic fluids with the exception of HCMV, where 3 samples labelled with asterisks (*) were present in both fractions of the amniotic fluid.

Viruses	Supernate of AF	Cellular sediment of AF	Total No. in AF	No. of viruses in blood samples
HCMV	**1+3***	**5+3***	**9+3***	**1**
HHV7	**0**	**8**	**8**	**1**
EBV	**1**	**3**	**4**	**1**
HHV6	**0**	**1**	**1**	**3**
HHV8	**5**	**0**	**5**	**7**
Total	**7+3***	**17+3***	**27+3***	**13**

* = viruses present in both supernates and cellular sediments

Seven samples of 96 were shown to contain human papillomaviruses. Association of herpesviruses and HPV are also shown in Table IV.

Table VI. Correlation of the presence of endotoxin and detected viral DNA in amniotic fluid samples.

Presence of viruses in amniotic fluid samples with detectable amount of endotoxin and in those with endotoxin concentrations below the level of detection. For control purposes the viruses found in the blood of the corresponding groups of mothers is also plotted into the right column (LR = low risk untyped HPV).

Viruses in endotoxin positive amniotic fluids and in the blood of the same mothers									
No. of sample	EU/ml	EBV	CMV	HHV6	HHV7	HHV8	HPV	Σ	Virus in blood
2	0.3								0
4	3.0						18/ LR	1+1	0
6	0.3								0
15	0.3								HHV8
18	0.3								0
19	3.0								0
39	0.3								0
41	0.3					HHV8	45	1+1	0
43	0. 03					HHV8		1	0
45	0.3								0
46	3.0		CMV					1	0
47	0.3								0
49	0.3								0
50	0.3								HHV8
52	3.0								0
Total 15/50		0	1	0	0	2	2+1	4+2/15 (26.7%	2/15 (13.3%)
Viruses in endotoxin negative amniotic fluids and in the blood of the same mothers									
28	Neg						Non - typed	1	EBV
37	Neg	EBV						1	0
42	Neg					HHV8		1	0
48	Neg					HHV8		1	0
Total 4/35								4/4	1/4
3, 5, 7, 8, 9, 11, 13, 14, 17, 22, 26, 30, 31, 32, 33, 34, 35, 36, 38, 40, 51, 53, 55, 56, 58, 59, 60, 71, 72, 73, 74 (Samples without endotoxin and virus)								4/35 (11.4%	1/35 (2.9 %)

Table VII. HPV genotypes and the limit of detection

HPV types in the amniotic fluid samples (7 samples of 96), sensitivity of the tests for different virus types tested is given in copy number in the second column. The presence of herpesviruses (4 samples of 7) are given in the fourth column. The right column indicates genogroups of mucosal types and the references (numbers in bracketts) which have been described in Hungarian patients (44, 45, 67, 115, 126, 199, 200, 220). Label "c" after virus specification indicates, that it could be detected from the cellular fraction of AF. A7 to A10 HPV genogroups dtected in Hungarian patients. The sensitivity of detection of HPV genotypes (6, 11, 16, 31, 33, 35) is given to show that the detection of common genotypes is sensitive enough to detect it if present in AF samples.

HPV type tested	Detectable HPV copy number	No. of Samples with HPV	Herpesvirus present in the sample	HPV genogroups (references on Hungarian prevalence)
6	160	None		A10 (45, 115,126, 199, 220)
11	74	None		A10(44, 115,126, 199)
16	970	None		A9(45,67,115,126,199, 200,220)
18**	70	(4A)	Non	A7(45, 67,115)
18**	70	(68A)	EBVc	A7(45, 67,115)
31	65	None		A9 (115)
33	160	None		A9(67, 115, 200)
35	135	None		A9(115,199)
39**	225	(63A*)	EBVc, HCMVc, HHV6c	A7
45**	305	(41A)	HHV8	A7(115,199)
58**	95	(63A*)	EBVc, HCMVc, HHV6c	A9(115, 199, 200)
58**	95	(91A)	None	A9(115, 199, 200)
62	Not done	(76A)	HCMVc	?(115)
Not typable		(28A)	None	?
Not typable (LR)		(4A)	None	?

4.7. Influence of endotoxins and viruses to the mean birth weight of neonates.

The mean weight of groups of neonates are given in Table VIII. Mean weight of the total of 106 neonates was 3280 g. That of newborns tested for all 6 viruses (99) was 3319 g. No significant reduction of mean weight was observed in the presence of endotoxin and/or viruses in the amniotic fluid except that of the six twins (Table V).

The results on the systematic examination of viruses in amniotic fluid samples revealed, that at least one third of the samples were containing viral DNA at the end of normal pregnancies.

Table VIII. Mean weight of newborn babies born from the mothers following healthy pregnancy and parturition tested for the presence of viruses and endotoxins. (*) Asterisk indicates, that one of the endotoxin positive amniotic fluid have been taken from a mother carrying twins. (**) Asterisks indicate, that two samples were taken from mothers carrying twins (***) Asterisks indicate that three samples were taken from mothers carrying twins.

Mean weight of newborn babies born from the mothers tested		
Group of neonates	**No. of babies**	**Weight (%)**
- total number of neonates	**106*** **	**3280 g (100.00)**
- boys	**61****	**3312.5 g (101.00)**
- girls	**45***	**3236.6 g (98.70)**
- twins	**6**	**2298 g (69.70)**
for endotoxins in AF	**53*** **	**3013.10 g (91.90)**
- tested negative for endotoxin	**37****	**2905.14 g (88.60)**
- tested positive for endotoxin	**16***	**3264.1 g (99.50)**
- tested for all 6 viruses	**99**	**3319.0 g (101.20)**
and found virus-positive	**23**	**3434.3 g (104.70)**
born from viremic mothers	**11**	**3000.0 g (91.50)**

5. Discussion

5.1. Seroprevalence of hepatitis viruses in Hungary before the introduction of the nation-wide mandatory HBV vaccination of school-children.

Comparison of the proportion of patients who had markers of active hepatitis B virus replication versus patients with other markers of active viral hepatitides became necessary because of the different preventive measures introduced in Hungary during the last decade. The perinatal transmission has been reduced below 1 % among the neonates of HBV-carrier mothers preventively treated with hyperimmune gamma globulin and active immunisation (131, 175).

The serological examination of diagnostic serum samples collected from hepatitis patients between 1993 and 2001 will serve as a basis for the measurement of the efficacy of mandatory nation-wide vaccination of school children about a decade later. The columns in Figure 12. summarise the data shown in Table III (Results) of indicate high rate of hepatitis B infections. These values should stepwise aproach to zero during the comming period of time. In connection with the serological survey double and triple infections with hepatitis viruses have been found. Growing evidence is accumulating that the clinical forms of viral hepatitis may be significantly modified in the case of coinfections (19, 51, 110, 111, 142, 163, 164, , 185, 186, 190, 219).

The results of the examination of 583 acute or active hepatitis patients suggested that double and triple infections occurred in this low-prevalence region of Hungary, too. In order to prevent dual infections or superinfections,

it is recommended to vaccinate all hepatitis patients after recovery or during remission (in the case of chronic disease) with the vaccines available against other hepatitis viruses (i.e. HCV patients with both HAV and HBV vaccines upon recovery or remission). Double or triple infections are not recognised under normal clinical conditions. Usually the routine diagnostic flowcharts are followed up to the first positive diagnosis.

The authors were also surprised that even triple infections may occur under natural conditions with hepatitis viruses. The possible source of infection might be the treatment of hemophiliacs with clotting factor. An other scenario might be the gamma globulin treatment of hepatitis A contact persons. Unfortunately the double blind collection of serum samples made it impossible to look for aetiology. Intravenous drug abuse has to be also taken into consideration as the source of multiple infections.

The preventive immunisation of the neonates initiated in 1995 will reduce significantly the number of healthy carriers (131, 144). The vaccination initiated in 2001 at the age of 14-15 years might result in the complete reduction of primary hepatitis B syndroms below 20 years of age after 2010.

The fact that the incidence of HBV and HCV positivity show different tendencies, suggest, that HCV is not transmitted mainly by the sexual route. In the prevention of HCV infections personal and hospital hygiene may play the most important role. The nosocomial HBV epidemic characterised by Szomor et al (202) might be a marker for the spread of viruses among the population under prolonged medical treatment.

Hepatitis A is exponentially decreasing in the subsequent age groups. According to the seroepidemiological survey performed in 1999-2000 (131, 159, 160), this decrease was associated with reduced seroprevalence of anti-HAV antibodies. Therefore, one has to recommend active immunisation for all persons, who are of increased risk of infection (i.e. contact persons of acute HAV patients) or suffering from any kind of liver damage (184).

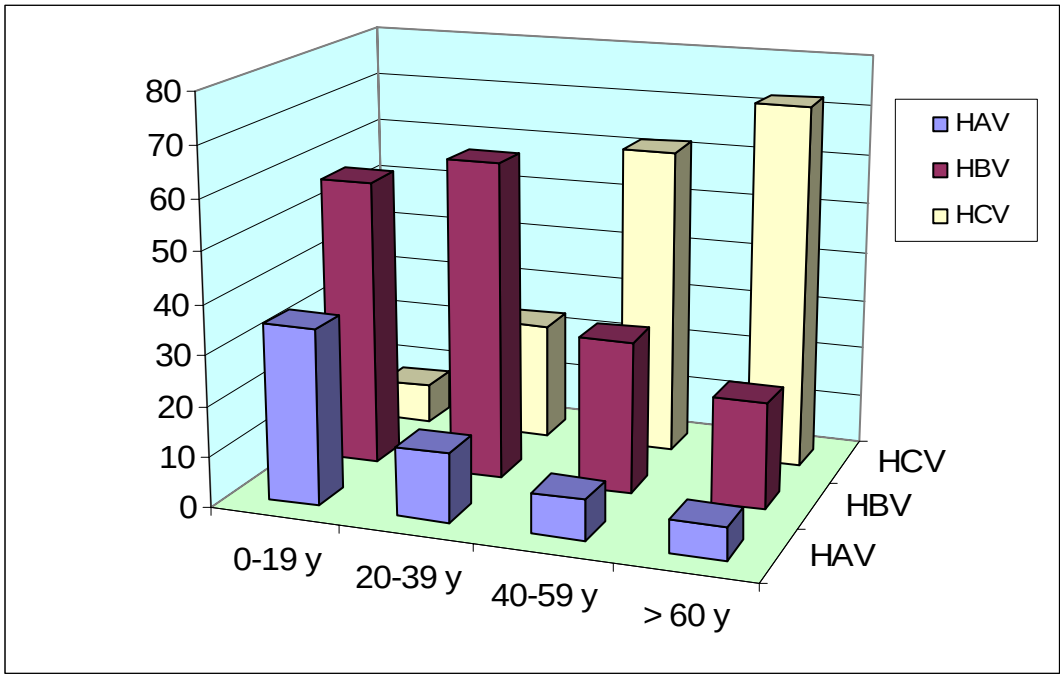

Figure 12. Per cent of viral hepatitis markers present in different age groups in the low prevalence, Southern Plain Hungarian Region as determined in serum samples drawn between 1993 and 2001 from patients with symptoms of hepatitis

Abbreviations: HAV: persons with anti-HAV IgM positivity, HBV: persons with HBsAg marker; HCV: patients with anti-HCV marker. (The data are taken from those listed in Table III.)

Double and triple infections occurred in the age group between 20 to 39 years. Probably because of the higher sexual activity and because of

the low rate of anti-HAV seropositivity (20 %) versus 60 and 80 % seropositivity of the older groups (159, 160).

5.2.) The presence of HBxAg and X-specific antibodies in HBsAg-positive persons.

Using the longest new recombinant protein (aa10-143) prepared (128) of HBxAg, the amount of HBxAg and anti-HBxAg has been titrated by the techniques standardised and elaborated recently (148, 149) in 106 serum samples of HBsAg-positive persons. The HBxAg antigen concentration was measured using monoclonal antibodies produced by the same authors. The results are not surprising, since HBsAg and antibodies, HBeAg and antibodies furthermore antibodies to HBcAg show similar behaviour in the three groups of patients examined.

Four of the people suffering from acute B hepatitis possess HBxAg above 500 ng/ml. Six others carried detectable amounts of HBxAg, but well below 200 ng/ml. Four of them had no detectable antigen in the circulation. Since it is not known when the blood samples had been drawn following the onset of the disease, one may conclude that all of them were HBsAg-positive. HBeAg is also produced later than HBsAg during the course of the disease followed by protracted immune response. The HBxAg seems to have similar kinetics of production and elimination than HbeAg. Four and five patients showed higher OD_{490} values of both IgG and IgM isotype antibodies. Probably these are the patients, who had no detectable antigen in their sera due to the immunocomplex formation (similar to the window period of HBsAg). The excess antibodies could be

probably only titrated. The kinetics of the production of anti-HBs IgM and IgG following vaccination (233) can be used for comparison.

In the case of the chronic hepatitis patients 19 had no detectable antigens, and 16 of them possessed excess antibodies, suggesting the existance of antigen-antibody complexes, too. The high proportion of patients with IgM antibodies suggest similarity of the behaviour of anti-HBc IgM in chronic hepatitis patients, being the diagnostic marker for their clinical disease. It has to be mentioned, however, that the damage of hepatocytes is required to induce IgM isotype antibody production in measurable concentrations to core antigens.

The very low mean concentration of HBxAg in the symptomless carriers indicate that the apoptosis of hepatocytes is very low in 6 persons. Six others had low antigen concentration except one of unknown reason. All of them possessed low levels of both IgG and IgM antibodies specific to HBxAg. The presence of immunocomplexes are also very probable. It might be possible, that the ELISA techniques are able to detect antibody-antigen complexes, too.

The systematic long term measurement of the HBV oncogenic X protein and the specific antibodies is an important duty for the future to recognise risk situation of the chronically ill or symptomless carrier persons. Recent examinations, however, revealed that the *HBx gene* has different behaviour within the cancer cells, and within other hepatocytes which replicate the virus. The mutational pattern of the *X-gene* were found to be different in liver cancer cells versus those found in the viruses circulating in the plasma of the patients (i.e. in hepatocytes actively

producing the virus). One might suppose, that the continuous expression of HBxAg itself may be a protein required by the replicating HBV, but without mutations does not represent increased risk for liver cancer in the symptomless carriers (35, 122).

Speculation is also possible on the question, whether the mutations detected in *X-genes* of the cancerous tissue were not simply a secondary consequence of the genetic lability of the host cells.

5.3.) Genotypes of hepatitis B virus in the Southern Plain Region of Hungary.

Accidentally a hospital epidemic had to be examined in the Department of Molecular Diagnostics at the national center for Epidemiology using PCR fragments of variable region of the hepatitis B genome. It seemed to be reasonable to test also DNA-positive samples from other regions of the country for control purposes. The samples collected from the Southern Plain region were found to belong to the „A" and „D" genotypes.

The historical contacts of the founding countries of the EU have invadors and refugies carrying all except "G" genotypes (Figure 13). Fortunately genotypes "A" and "D" were found to possess less oncogenic property in contrast to the „C" genotype prevalent in the Far-East and the United States (121, 122, 129, 235).

The importance of the preventive measures for the eradication of HBV from the Hungarian population is emphasised by the fact that this

nation wide programmes will prevent the spread of more oncogenic genotypes into Hungary.

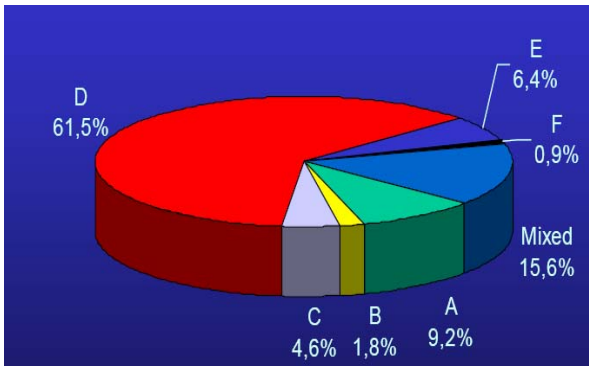

Figure 13. HBV genotypes in the European Union. 109 DNA samples had been sequenced in Luxembourg

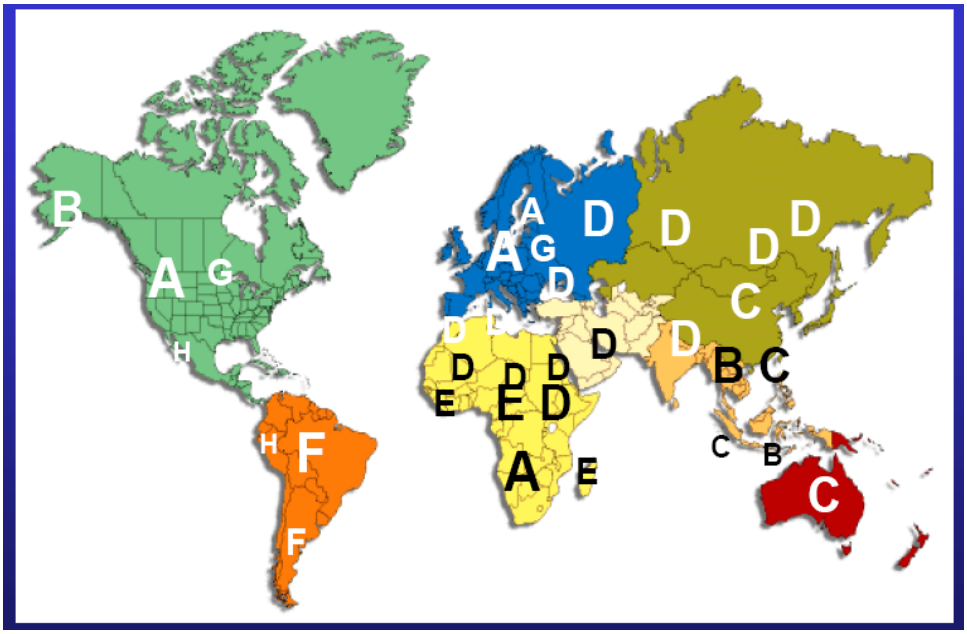

Figure 14. Global distribution of hepatitis B genotypes (A to H). The genotypes identified up to now in Hungary and also in the Southern Plain Region of the country were "A" and "D".

In the population of the European Union (Figure 13) the "D" genotype is the most prevalent. Genotype "A" and recombinant genotypes are present of equal frequency. Genotypes "B", "C", "E" and "F" have been also detected. The later, however, had not been identified in Hungary. One has to test in the future the Chinese minority being the

probable source of the “B and C” genotypes (Figure 14). The dissemination of these genotypes in the country might result in the groing number of hepatocellular cancer tw decates later.

5.4. Absence of external contamination of amniotic fluid samples

Only trans-vaginal sampling was approved by the competent Review Board, it had to be performed very carefully. The results were analysed first from the viewpoints of contamination with vaginal secretions.

The presence of enteroviruses (99) herpes simplex virus type 2, papillomaviruses, HCMV, HHV8 and EBV has been shown in vaginal secretions previously (14, 31, 59, 89, 119, 228). In order to exclude retrograde contamination 96 samples were tested for the presence of HSV2 using nested PCR. The sensitivity of the procedure was 200 copies/sample (experiments not documented). No HSV2 DNA was detected in any of the AF samples.

HHV8 may be present in vaginal secretions (31), but in AF samples only 5 positive results were obtained in contrast to 7 positive results in blood samples. It has been also shown previously that the presence of antibodies in vaginal secretions cannot prevent detection of homologous DNA (220, 221). The DNA remains analtered in immunocomplexes under appropriate storage conditions. The macroscopic observation of the AF samples excluded such contamination with maternal blood which might contaminate the samples with viruses.

Papillomaviruses were found in secretions and tumours of Hungarian women by previous authors (45) and the prevalence of types was HPV6 (12 %), HPV11 (8%), HPV16 (9%) and HPV18 (6%). About half of 102 positive vaginal samples tested by the same group (115) contained HPV16 (46 %). HPV6 (12%), HPV31 (10%), HPV 18 and 52 (6-6%), HPV33 (5%) and HPV types 11, 56, 35, 45 and 44 (1-3 %). In addition 12 samples contained double infections (115). HPV 6 and 16 were found to compose more than the half of types when smears of healthy women had been tested (220). As shown in Table VII. HPV6, 11 and 16 could not be detected at all in AF samples tested in tour work. The minimum copy number of these genotypes detectable by the technique used is included in to Table VII. The data show that the sensitivity is sufficient for the detection of other genotypes, too. HPV 18 was identified in 2 of 96 samples, and 2 of 96 samples showed double infections. The unusual composition of anogenital types in AF indicates again no contamination of AP with vaginal secretion.

The fact, that the majority of herpesviruses (except HHV8) detected in the cellular sediments of amniotic fluid (Table V) was also a hard argument against contamination, since contaminating viruses would be detectable mainly in the supernates. There were only two of 27 pregnants who had viruses both in AF and blood. In spite of the fact that the tubes containing AF and full blood were tightly fixed to each other with adhesive tape during storage, the two positive sample-pairs contained different viruses (Table IV).

Finally it is very improbable that measurable amounts of endotoxins could be transferred from the surface of tubes into 4 to 10 ml volumina of the samples. It is concluded that the viruses detected were not the result of retrograde contamination during sampling.

5.5. Viruses and endotoxin in AF and total blood samples of healty pregnants

Fifteen of 50 amniotic fluids taken at birth contained endotoxin between 0.03 to 3.0 EU/ml concentrations. The presence of endotoxin has been suggested to cause preterm delivery (39, 173, 174). The existence of endotoxin-neutralising protein(s) identified in human placenta (107) might suggest that only surplus amounts or unusual chemical forms of the endotoxin may pass the placenta just before delivery. The successful termination of the pregnancy was probably the result of increased resistance of the fetus due to toll-like receptors (66). It has been observed, that AF samples without live bacteria may be associated with premature labor and delivery due to inflammation detected by elevated cytokine concentrations (50, 98, 197, 227). The presence of endotoxin in healthy pregnancies might be associated with post-natal impairment of organs due to prolonged exposure to endotoxins.

The virus content of endotoxin positive and negative AF samples are shown in Table VI. Four of 15 endotoxin positive samples contained 6 viruses. Four of 35 amniotic fluids with undetectable endotoxin content proved to be virus positive. The blood samples of the corresponding mothers, however, resulted 2xHHV8 of 15 pregnancies with endotoxin

positive AF samples. The number of viruses was 1 of 35 in the blood of the endotoxin-free group. Differences in virus content of endotoxin-positive and negative AF samples were not significant in any of the combinations tested.

The endotoxin tests have not been continued, since the tests have been very expensive and the Food and Drug Administration (FDA) approved the use of parenteral vaccines containing 100 Endotoxin Unit/ml contamination in parenteral vaccines. The finding indicated that the highest concentration in amniotic fluid was 33 times lower than that allowed for killed or subunit influenza vaccines. It is easy to undesrtand, that no adverse effects (induction of cytokines, or tumor necrosis factor) can be seen and the development of the fetus had not been impaired at the measured endotoxin concentrations.

The published data on possible counteracting mechanisms and further research on the elimination of endotoxin during pregnancy would require further research.

5.6. Reactivation of lymphotropic herpesviruses

Reactivation of herpesviruses and papillomaviruses during pregnancy have been shown earlier. Several days after delivery the viruses cannot be detected in the secretions of the mother, due to the recovery of immune modulation (45, 84, 221). The selective behaviour of different herpesviruses had been recognised by previous authors (84, 153). The lymphotropic herpesviruses were probably reactivated in the immune system and transfered into the placenta and fetal tissues with some delay

after reactivation. The presence of cytokines may reactivate monocytes, too (143). The reactivation and replication of HCMV may be accelerated by these phenomena. The disproportionate distribution of viruses in AF and blood samples suggests that HCMV, HHV7 and EBV have been reactivated first, transported to the AF and the viremia or presence of virus carrier cells in mothers blood have decreased thereafter (Table V). For HHV7 a similar phenomenon had been put forward a few years ago (153, 228). Reactivation of HHV6 and HHV8 occurred probably slower or the transfer to the AF was protracted. The prevalence of these viruses in blood was higher than that in the AF (10, in contrast to 6). In the case of HHV6 the latter behaviour has been also suggested previously (153).

The relatively high number of HHV8 positive AF supernates suggested the recent transfer of the virus into the fetal compartment. The presence of the majority of HHV7, EBV, HHV6 and HCMV in the cellular sediment of AF (17 viruses in contrast to 7 in the supernates) might indicate their long term presence in fetal tissues, which enabled their trapping by cellular debris or transfer into meconium. HCMV of 3 pregnants was present in both supernates and cellular sediments of AF. This observation might indicate an intermediate position or protracted transfer through the materno-fetal barrier.

Pregnant #63 had HHV6 in the AF, and pregnant #97 in the blood, but no HHV6-specific antibodies could be detected in their blood. Probably 2 of 104 mothers were immunotolerant to HHV6 or had an acute infection or reactivation just before parturition. Further examinations

might reveal, whether post-partum immunotolerance can be induced by viruses passing the placenta, similarly to the hepatitis B surface antigen.

The profound differences of humoral immune response to EBV, and HCMV in contrast to that of HHV8 being significantly lower in myeloma multiplex patients (21) might be an indication that the antigens met the fetal immune system preceeding birth.

Co-infections have stimulated replication of viruses in cells after dual infection (211, 212). Even abortive replicative cycles of HCMV and EBV has been shown to be completed upon the influence of interleukins and TNF-β1 (15, 16). These factors might be responsible for the presence of 2 to 5 different viral genomes in 8 of the virus positive AF samples.

5.7. *Papillomaviruses in AF samples*

Seven AF samples of 96 were shown to contain papillomaviruses (Tables IV, VII and Figure 15). All, except genotype 39 had been found in publications reporting genotypes in secretions or benign and malignant tumours of Hungarian patients (45, 67, 115, 126, 199, 200, 220, 221 and Figure 15.). All except 1 of them (patient #28) were identified to be anogenital types.

Our data have confirmed previous results (9, 10, 77, 78, 84, 133, 134, 173, 178) concerning vertical transmission of viruses during fetal development in spite of the presence of maternal antibodies. Serological detection of possible immunotolerant virus-carriers might be the consequence of this rare event. Further experiments will be required to

detect whether such partial immunotolerance might contribute to cancer development in addition to oncogenic potential of the virus concerned.

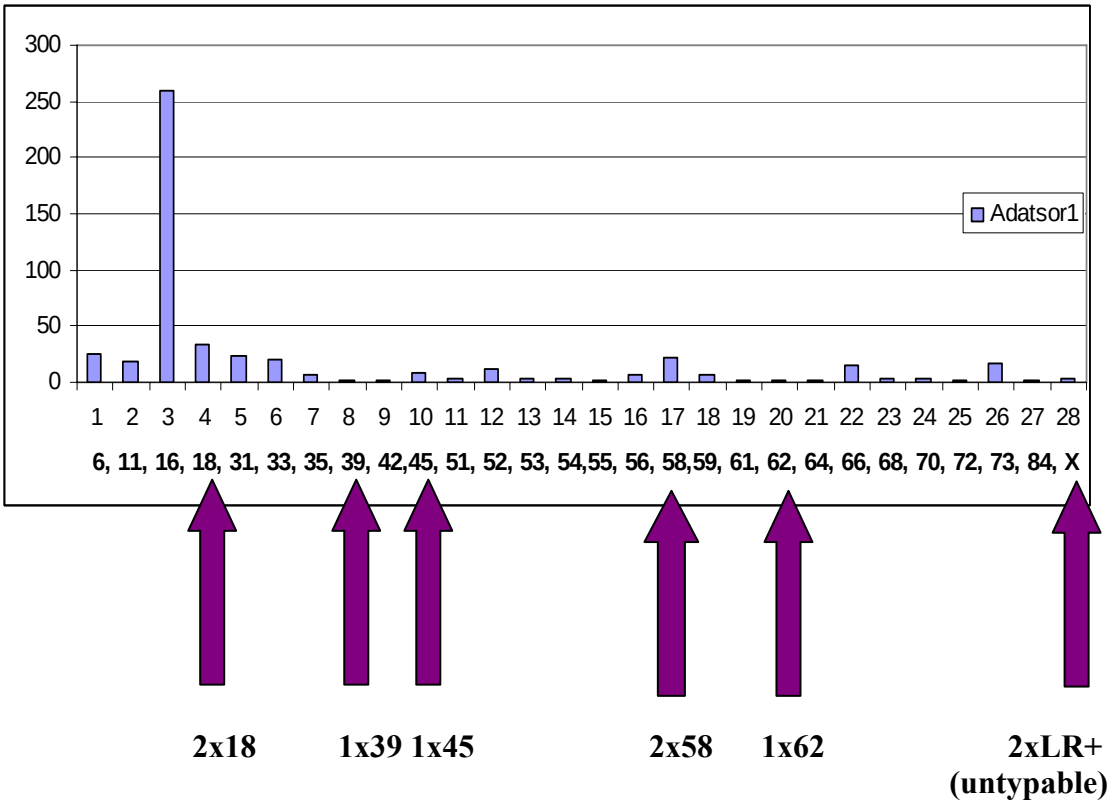

Figure 15. **Human papillomavirus types identified in Hungarian patients** (499) and the types detected in the amniotic fluids (arrows with No. of types identified). The HPV types identified in Hungary are shown below the serial numbers of the columns (45, 46, 47, 48, 49, 68, 93, 115, 126, 155, 161, 199, 200, 220, 221).

Presence of non-replicating HPV-DNA in neural structures and vascular endothelial cells (67) expressing exclusively early proteins, but no structural ones without any immune response might suggest both fetal infection and immunotolerance. The neonatal presence of developed condyloma accuminatum (172) may also suggest prenatal transfer of virus or virus infected cells through the placenta.

5.8. Effect of the endotoxin and viruses in the amniotic fluid to the mean birth-weight of neonates

No significant difference could be detected in the mean weight of babies born from mothers with or without endotoxin and viruses in AF. Future research might reveal medical importance of the findings. Further follow up of children is recommended to clarify delayed "post partum" consequences of expositions during fetal life.

The presence of defence mechanisms preventing endotoxin effects i.e. increasing of the expression of Toll-like receptor 4 (CD14) with time have been shown to reduce sensitivity of the fetus to endotoxin effect (66, 87).

Endotoxin-neutralizing antimicrobial protein in the human placenta eliminating endotoxin effects in the amniotic fluid at the final stage of healthy pregnancy have been also identified (107). These mechanisms might contribute to the published rescue of 6 newborn babies out of 18 who have been born without any clinical consequences in spite of the presence of endotoxin in the amniotic fluid (60). The gradually increasing resistance of fetal tissues until delivery prevented probably impairment of neonates against noxae at the very end of the pregnancy.

6. Conclusions and perspectives of the results

A systematic examination of other different viruses latently present in the human organisms should be carried out in the future. Human adenoviruses, human parvovirus B19, HTLV-I and II, hepatitis G virus, TT virus, polyoma viruses might be the candidates for such examinations.

The presence of TT virus had been detected already in vaginal secretions of the pregnant women (34).

In the case of the transplacental transmission, the fetal immune system might develop immunotolerance to certain viral proteins or early proteins. A sytematic search could be able to differentiate individuals with and without partial immunotolerance to certain viral antigens in the population.

Such a partial immunotolerance might result in a reduced number of cytotoxic T8 cells or impairment of their activity against virus-infected and virus carrier cells. This phenomenon would facilitate tumour formation. Between 1972 and 1979 researchers of the International Cancer Center in Lyon tested 42,000 children for presence of Epstein-Barr virus specific antibodies. Fourteen children developed Burkitt's lymphoma during the five years of the observation periode up to 1979 (54). The serological examination of the tumour-bearing children revealed that the antibody titers against viral structural proteins have been several times higher in their sera than that in the children free of sarcoma in the surroundig population. No difference has been found, however, in the antibody titres specific to EBV early proteins or EBNA (54, 71). This different behaviour of the antibodies is peculiar, since the tumour cells harboured EBV DNA. One would suppose, that the antibodies against non-structural proteins were also high due to the large masses of tumour tissue.

The authors observed that all children with Burkitt's lymphoma had been seroconverted before the 6^{th} month of age. This finding might

indicate a perinatal or transplacental infection resulting in the low reactivity of the immune system to non-structural antigens.

The serological methodology of the research team in the seventies has not been as sofisticated and sensitive than nowdays. The selective examination of immune response to structural and non-stuctural proteins could reveal differences in people who acquired the infections transplacentally at the end of pregnancy or after birth.

Non-responsiveness of a considerable proportion of health workers to hepatitis B recombinant vaccines might be also the consequence of the transient contact during fetal life or perinatal infection to HBV surface antigen without a successful productive infection (40). Without the impairment of the immune system the malignant lymphomas carrying EBV virus were shown not to develop. The immunotolerance detected here might be one of the factors, which might contribute to the molecular changes observed by many authors and reviewed recently (109).

The different behaviour of the immune response in myeloma multiplex patients to antigens of HCMV, EBV, HHV6 in comparison to KSHV might also indicate perinatal exposure. Unfortunately the early and structural antigens have not been examined separately yet (41).

Recently several new concepts of tumour genesis have been put foreward and reviewed (46, 109, 146). Several unresolved problems might be explained using the idea that a small proportion of individuals' immune systems had direct contact to viral antigens in their antenatal life.

As far as the endotoxin content of the amniotic fluid is concerned, the measured concentrations are well below the concentration aproved by the FDA for parenterally injected vaccines.

An alternative explanation for the low levels of endotoxin can be the effective activation of mechanism counteracting the transfer or prolonged presence of them in the amniotic fluid .

The new observations of the works are the following:

1.) A survey has been completed on samples taken before the introduction of the mandatory vaccination in 2001 in order to possess data for comparison on patients with markers of acute, chronic or symptomless carriers of classical hepatitis viruses in the Southern Plain Region of Hungary.

2.) Double and triple hepatitis infections have been observed.

3.) It has been suggested, that the available vaccines have to be offered for all patients suffering from any of the hapatitis infections after recovery or during remission.

4.) The pattern of the HBxAg and anti-HBx antibodies is similar to the behaviour of HBeAg and anti-HBe antibodies indicating the presence of specific immunocomplexes during the history of hepatitis B infections. No profound differences could be observed between acute, chronic HBV diseases and in the serological findings of symptomless carrier persons.

5.) DNA of five viruses have been systematically tested in both the amniotic fluid and maternal blood of pregnants at term . One third of the amniotic fluids were found to contain viruses, and one third of the

amniotic fluids tested was found to contain measurable amounts of endotoxin.

6.) The endotoxin concentration was very low in the samples, and exerted no influence to the mean body weight of the neonates.

7.) The viruses were distributed unequally in the low speed supernate and meconium and/or cell debris of the amniotic fluid samples.

8.) Significant difference could be detected in the appearance of the viruses in the maternal blood and in their transfer into the amniotic fluid.

9.) The consequences of the contact of fetal immune system and viral antigens have to be taken into consideration in future diagnostic work with patients suffering from different lymphotropic herpesviruses..

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10. Abbreviations:

ADAM	A disintegrin and metalloprotease
AIDS	Aqcuired immunodeficiency syndrome
AP	Alkalyne phosphatase
ATCC	American Type Culture Collection
BCBL-1	Body cavity B-cell lymphoma No. 1. clone
CK	Cytokeratin
DC	Dendritic cells
DIG-Ap	digitoxigenin-labelled alkaline phosphatase
EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus
EDTA	Ethylene diamine tetra-acetate
EIA	Enzyme immuno assay
ELISA	Enzyme linked immunosorbent assay
Endotoxin	Lipid A, LPS
EVER1 and 2	Genetic mutations of *Epidermodysplasia verruciforme*
FCE	Fetal capillary endothelium
FcR	Fc-receptor on cell membranes
FDA	Food and Drug Administration
FV	Floating villi
gB to gI	Glycoproteins of herpesviruses
GST	Glutathion S-transferase
HAV	hepatitis A virus
HBcAg	„core" antigen of HBV
HBeAg	„Pre-core" antigen of HBV
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HBx	oncogene of HBV
HBxAg	oncogenic protein of HBV (Hepatitis B X-protein)
HCV	hepatitis C virus
HCMV	Human cytomegalovirus
HEV	hepatitis E virus
HHV6	Human herpesvirus type 6
HHV7	Human herpesvirus type 7
HHV8	Human herpesvirus type 8 (See KSHV)
HIV1	Human immunodeficiency virus type 1
HO	Hofbauer macrophages
HPLC	High Pressure Liquid Chromatography
HTLV-I	Human T-cell leukemia virus type I
IC	Internal control for HPV quantitation
ICAM-I	Intercellular adhesion molecule type I
IDT	Integrated DNA Technologies
IgG	Immunoglobulin G
IgM	Immunoblobulin M
IL-6	Interleukin-6
IL-6-R	Receptor for interleukin-6
KSHV	Kaposi's sarcoma herpesvirus (HHV8)
LAL	Limulus amoebocyte lysate

L1F	Forward primer set for L1 gene of HPV genotypes
L2R	Reverse primer set for L2 genes of HPV genotypes
LCR	Long Control Region of HPV DNA
LPS	Lipopolysaccharide (Lipid A), endotoxin
MLR	Mixed lymphocyte reaction
MOAB	Monoclonal antibody
NCE	National Center for Epidemiology
NPHMOS	National Public Health and Medical Officer Service
nt	Nucleotide
5'-NTR	Non-translated region of picornaviruses
$O.D._{.nnn}$	Optical density at the indicated wavelength
OPD	o-phenylene-diamin
QCMD	Quality Control for Molecular Diagnostics
p24	polypeptide of 24,000 relative molecular mass
PCR	Polymerase chain reaction
RPMI	Tissue culture medium for cultivation of lymphocytes
RT	Reverse transcriptase
SDS	Sodium dodecyl sulphate
SEN	short variant of TTV virus
ST	Syncytiotrophoblast
X-GST	Fusion protein of HBxAG-and GST
TNF	Tumor necrosis factor
TTV	„transfusion transmitted virus" (old nomenclature)
VZV	Varicella zoster virus

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