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Detection of latent human cytomegalovirus in organ tissue and the correlation with serological status

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Abstract. The presence of human cytomegalovirus (HCMV) genome in spleen tissue was studied by using DNA hybridization techniques in seropositive and seronegative organ donors without clinical or laboratory confirmed HCMV infection. The serum samples of these patients were screened by latex agglutination test (LA) and enzyme linked immuno sorbent assay (ELISA) for the presence of HCMV antibodies, and confirmed by immunoblotting technique (IB). For the detection of HCMV sequences in spleen tissue dot blot DNA hybridization (DBH) using probes derived from immediate-early and late regions (ES and BH fragment respectively) of the HCMV genome were used. Samples positive in DBH were further tested by in situ DNA hybridization (ISH) using the ES probe. The number of spleen tissue specimens positive for HCMV nucleic acids indicated that HCMV may be present in human beings, even without serological evidence.

Key words: Human cytomegalovirus – Latency – Hybridization – Antibodies

Human cytomegalovirus (HCMV), a member of the herpesvirus family, is an ubiquitous human viral pathogen. Infection with HCMV is usually asymptomatic in the immunocompetent host, but can result in a dramatic disease in immunosuppressed patients [15]. After primary infection the virus persists in the host as a chronic or latent infection, which can periodically reactivate to an active infection. HCMV is known to be transmitted through blood transfusions [1, 24] and transplanted organs [10, 33] which can result in a severe HCMV disease, especially in immunosuppressed and seronegative patients [3, 22]. Several studies indicate that in HCMV seropositive healthy individuals latent infection can be demonstrated by the presence of HCMV antigens. This is done by immunohistochemical techniques or by the detecting the presence of HCMV genome by DNA techniques in smooth muscle cells of arteries and in different cell types of various organ tissues, including kidney, liver, lung and spleen [12–14, 21, 25, 32, 34]. The presence of HCMV specific antibodies as evidence of past or present infection is the most valuable indicator of a potentially infective donor [9, 20]. However, there is some doubt if all seronegative donors are really free from previous HCMV infection, with the possibility of the presence of a latent virus [8, 28, 29, 31].

This suggestion prompted us to analyze organ tissue derived from seronegative organ donors for the presence of HCMV genome in organ tissue. To detect HCMV nucleic acids in organ tissue in our study spleens were subjected to DNA hybridization techniques. Spleen tissue was used for several reasons: firstly, the availability of spleen tissue of organ donors, secondly, this tissue has been demonstrated to be a site of latency for HCMV [32] and for murine CMV [26], thirdly, the high density of cells in spleen tissue and fourthly, the immunocompetent function of this organ.

For these investigations spleen tissue from seronegative donors was first analyzed by dot blot DNA hybridization (DBH), followed by the more specific in situ DNA hybridization (ISH) of the DBH positive samples for localization of the latent virus. Spleen tissue from seropositive patients (kidney donors) known to contain latent HCMV in their arterial walls [12], served as a positive control group for all DNA hybridization techniques. For the detection of HCMV antibodies in the serum samples the latex agglutination test (LA), which is used in most laboratories, and the enzyme linked immuno sorbent assay (ELISA), were used [2, 11]. To confirm the HCMV antibody negative status of the patients the very sensitive immunoblotting technique described by Landini et al. [17] was performed.

Materials and methods

Selection of patients and specimens. Patients whose cause of death was a non-HCMV realted disease i. e. organ donors (trauma victums and patients whose cause of death was a sudden cardiac arrest), and whose spleen tissue (as routinely paraffine embedded) was available

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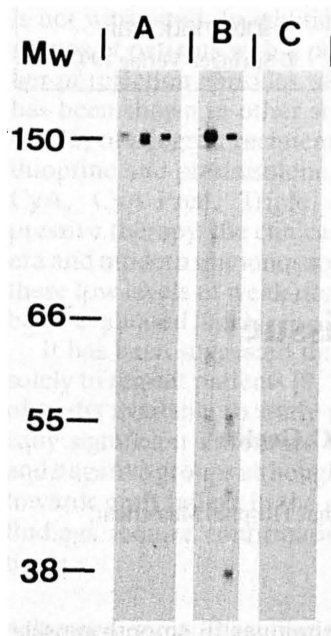

Fig. 1. Results from immunoblot detection of antibodies to HCMV proteins in sera of healthy persons.
(**A**) Three lanes of sera with only positive reaction for the 150 kD protein.
(**B**) Two lanes of sera with specific antibody reactivity for several proteins.
(**C**) Three lanes of sera without antibodies reacting to HCMV proteins

were included in this study. Patients suffering from immunosuppressive disease or undergoing immunosuppressive therapy were strictly excluded.

Latex agglutination (LA). The latex agglutination test (Becton Dickinson Microbiology Systems, Cockeysville, USA) was performed according to the instructions of the manufacturer. Undiluted serum samples were screened on a disposable card slide and allowed to agglutinate with the HCMV antigen coated latex particles.

Enzyme linked immuno sorbent assay (ELISA). The procedure for ELISA has been previously described [18, 19]. Briefly, antigen material was derived from HCMV (AD169 strain) infected human embryonal fibroblasts (HEF). The sera to be tested were diluted 1:200 and allowed to react with the antigens for 2 h at 37 °C. Horse radish peroxidase-conjugated, goat polyclonal antihuman IgG (Institute Pasteur, Paris, France), diluted 1:1000 served as second antibody. The incubation with this conjugate was done for 2 h at 37 °C. This was followed by adding substrate (o-phenylene diamine and hydrogenperoxidase) to the wells. The optical density at 492 nm was measured in a Titertek Multiscan by an ELISA reader (Flow Laboratories, Irvine, Scotland).

Immunoblotting technique (IB). The IB was performed essentially as described previously [7, 16]. Briefly, purified HCMV (AD169 strain) virons were subjected to sodium dodecyl sulfate polyacrylamide gel electroforesis (SDS-PAGE) and the separated polypeptides electrically transferred to nitrocellulose sheets (Schleicher and Schuell, Dassel, Germany). The human sera, routinely diluted 1:100, were incubated overnight at room temperature with the transferred proteins, followed by an incubation with rabbit antihuman IgG labelled with horse radish peroxidase (Dako, Glostrup, Denmark). The immune complexes were visualized by staining with 4-chloro-1-naphthol (Biorad, Richmond, California, USA). A serum specimen was considered positive for HCMV by immunoblotting if antibodies reactive to one or more of the major structural proteins having relative mobilities of 150, 82, 66, 55, 38 or 28 kiloDaltons (kD) were present.

DNA extraction. DNA was isolated as described previously [5]. Briefly, 5 tissue sections of 10 µm "thick" with an average surface of 1 cm^2 were deparaffinized by xylene followed by ethanol (96%) washes. Subsequently, the tissue was vacuum dried and digested overnight at 55 °C in a mixture of 50 mM Tris (pH 8.5), 1 mM EDTA and 0.5% Tween 20 with 200 µg proteinkinase K in a final volume of 400 µl. To purify the DNA, a part of the lysate was added to guanidine thiocyanate-lysisbuffer (Fluka Chemie AG, Buchs, Switzerland) and diatom suspension (Sigma, St. Louis, USA). Finally, the nucleic acids were released from the diatoms in destillated water. The DNA concentration was determined spectophotometrically by measuring the extinction at 260 nm.

Dot blot hybridization (DBH). Aliquots of 5 µg cellular DNA were spotted on nitrocellulose filters (Amersham, Buckingshamshire, England). Hybridization was performed essentially as described previously [12]. To detect HCMV specific sequences the filters were hybridized with the major immediate early region of the HCMV genome (ES fragment) and the late region of the HCMV genome (BH fragment). The fragments were ^{32}P labelled in vitro by using a random primed DNA labelling kit (Boehringer, Mannheim, Germany). After hybridization the filters were washed under medium stringent conditions. The final wash-step was in 0.1 × SSC, 0.5% SDS for 45 min at 50 °C. Hybridization was visualized by exposing the filters to a Kodak X-omat film (Eastman-Kodak Company, Rochester/New York, USA) for 4 days at −70 °C.

In situ hybridization (ISH). For localization of the cell type(s) involved in a site for HCMV latency in spleen tissue the dot blot hybridization positive specimens were subjected to the specific ISH procedure with the ES fragment, derived from the immediate early HCMV genome. ISH was performed on 4 µm "thick" tissue sections as described previously [12]. For ISH the ES probe was labelled with biotin by incorporation of biotin-11-dUTP (Bethesda Research Laboratories) using a random primed hexanucleotide procedure (Boehringer, Mannheim, Germany). The hybrids were visualized by using the BLU gene ™kit (Bethesda Research Laboratories). The specificity of the probes was tested on DNA extracted from uninfected HEF monolayers and HEF monolayers infected with HCMV (AD169 strain). Other human herpes viruses (HSV, VZV, EBV, HHV-6) were used as controls.

Results

Selection of patients and specimens

As positive control 5 seropositive organ donors, with a mean age of 39 years, known to contain HCMV nucleic acid sequences in their arterial walls [13] were included in this study. Also included were 45 HCMV seronegative patients from whom splenic paraffine embedded tissue blocks were available. This group consisted of 24 men and 21 women, with a mean age of 49 years. Most patients were trauma victims ($n = 26$), while the other patients died of sudden cardiac arrest ($n = 19$).

Serology

A total number of 87 serum samples were screened by LA and ELISA for the detection of specific HCMV antibody. Using LA, 42 of 87 sera were positive and 45 of 87 serum samples were negative for antibodies to HCMV. Using ELISA, 41 of 87 sera were positive and 46 of 87 specimens were negative for HCMV antibodies. Sera from the positive control group gave positive reactions for specific HCMV antibodies using LA and ELISA. By immunoblotting, all seropositive control sera, as determined by LA as well by ELISA, reacted with the 150 kD HCMV structural polypeptide and some of them reacted with several HCMV structural proteins with the apparent molecular weight of 82, 66, 55, 38 and 28 kD. However, these reactions were of a lower intensity in comparison with the reaction with the 150 kD polypeptide, as demonstrated in Fig. 2. Of all the 45 LA and ELISA seronegative specimens, no reactivity was observed to any of the specific structural viral proteins.

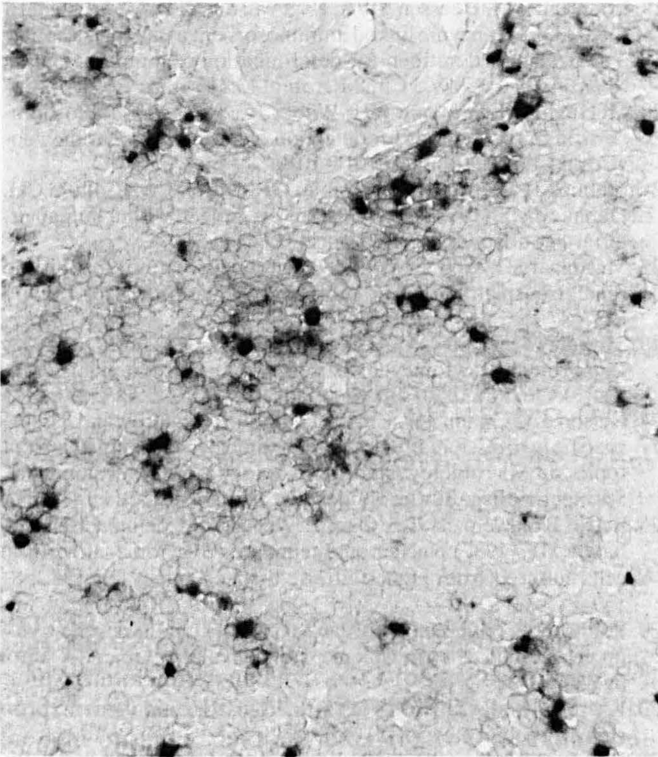

Fig. 2. HCMV DNA was detected in the nuclei of splenic red pulp cells of a section of a spleen from an organ donor. The slide was hybridized in situ with the HCMV subgenomic ES fragment labelled with biotin-11-dUTP. The *dark spots* represent the hybridization

Hybridization

Spleen tissue of the seronegative donors was tested for the presence of HCMV related sequences using DBH and ISH. Using DBH, 9 out of 45 (20%) tested spleen specimens were positive for the presence of specific HCMV nucleic acids both with ES and BH fragment. Testing these 9 HCMV antibody seronegative spleen tissues by ISH with ES probe to determine the latent HCMV positive cells, in 7 out of these 9 (77.8%) specimens a specific hybridization signal for the presence of HCMV was obtained in the nucleus of perifollicular red pulp cells (Fig. 2). This localization was in concordance with the ISH results of the seropositive control spleen specimens. Specimens positive by DNA hybridization techniques were randomly distributed over the seronegative patients. Of the control group 5 out of 5 spleen tissues gave positive result using DBH as well as ISH.

To exclude crossreactivity with cellular DNA or other human herpesviruses the ES and BH probe were tested on DNA extracted from infected HEF monolayers. No crossreactivity was observed, while DNA obtained from HCMV (AD169 strain) infected HEF always showed positive results.

Discussion

In this study we showed the presence of latent HCMV in splenic tissue in seropositive individuals. In the nucleus of the red pulp cells of the spleen HCMV positive ISH was found. This observation confirmed the results of other laboratories indicating that spleen tissue can be a site for latency of HCMV [32]. This localization is also in agreement with in vivo animal experiments; perifollicular red pulp cells are infected in acute infections [30] and are demonstrated to be a site for latency in CMV infected animals [26]. As has been shown in several other studies [6, 9, 11, 20], it is important to use a dependable serological assay for screening blood and organ donors to achieve a reduction of transmitted HCMV infection and HCMV disease in seronegative recipients at risk of primary infection. However, there is some debate about seronegative donors being free from previous HCMV infection and the possibility of presence of latent virus [10, 28, 29]. Using the DBH technique we found that in 9 out of the 45 (20%) patients HCMV genome could be detected. The moderate number of HCMV seronegative normal individuals having HCMV nucleic acids detectable in the spleen tissue as demonstrated in this study could be explained by one of the following mechanisms. Firstly the HCMV antibodies could be absent in these patients due to some deficiency in antibody production to viral protein(s) [29]. Secondly, it is generally accepted that after HCMV infection, antibodies are developed and these antibodies persist throughout life. The question could be asked if this persistence is obtained by frequent (local) reactivation of the latent virus. If this is the fact than it is possible that without this reactivation the level of antibodies decline during life reaching an undetectable level (so called seronegativity) [23]. If this hypothesis is correct than seronegative patients could harbor latent HCMV in their organs. An explanation for this phenomenon is the suppression of viral expression due to an increased level of methylation of the viral genome [4, 27]. Thirdly, although the DNA probes used in this study were very specific for HCMV, the possibility of detecting an (unknown) (Herpes) virus with nucleic acid sequences in its genome similar to the immediate early and late regions of the HCMV genome exists.

Further investigations are needed to elucidate the presence of latent virus in seronegative humans and to detect factors leading to seronegativity due to suppression of the immune response in the immunocompetent host.

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