Human cytomegalovirus in the vascular tree and other organsystems

Citation for published version (APA):

Hendrix, M. G. R. (1998). *Human cytomegalovirus in the vascular tree and other organsystems*. [Doctoral Thesis, Maastricht University]. Universiteit Maastricht. <https://doi.org/10.26481/dis.19981202mh>

Document status and date:

Published: 01/01/1998

DOI:

[10.26481/dis.19981202mh](https://doi.org/10.26481/dis.19981202mh)

Document Version:

Publisher's PDF, also known as Version of record

Please check the document version of this publication:

- A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
- The final author version and the galley proof are versions of the publication after peer review.
- The final published version features the final layout of the paper including the volume, issue and page numbers.

[Link to publication](#)

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal.

If the publication is distributed under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license above, please follow below link for the End User Agreement:

www.umlib.nl/taverne-license

Take down policy

If you believe that this document breaches copyright please contact us at:

repository@maastrichtuniversity.nl

providing details and we will investigate your claim.

Human Cytomegalovirus in the vascular tree and other organsystems

1998, M.G.R. Hendrix, Enschede, The Netherlands

ISBN 90-9012216-8

Printing: FEBODRUK BV offset drukkerij, Enschede.

The publication of this thesis was financially supported by by Laboratorium microbiologie Twente Achterhoek, and the Medisch Spectrum Twente.

Human Cytomegalovirus in the Vascular tree and other Organsystems

Proefschrift

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
op gezag van de Rector Magnificus,
Prof. Dr. A.C. Nieuwenhuijzen
volgens het besluit van het College van Dekanen,
in het openbaar te verdedigen
op woensdag 2 december 1998 om 14.00 uur

Door

Matheus Gerardus Ronald Hendrix

Geboren te Maastricht op 28 november 1958

Promotor:

Prof. Dr. C.A. Bruggeman

Beoordelingscommissie:

Prof. Dr. P.J.E.H.M. Kitslaar (voorzitter)

Prof. Dr. M.J.A.P. Daemen

Prof. Dr. F.C.S. Ramaekers

Prof. Dr. Teuchy (LUC Diepenbeek, België)

aan mijn ouders,
aan Esther,
aan Elke en Jorn

Contents

List of abbreviations

Chapter 1	Introduction. Aims of the study
Chapter 2	General introduction
Chapter 3	The presence of cytomegalovirus nucleic acids in arterial walls of atherosclerotic and nonatherosclerotic patients
Chapter 4	High prevalence of latently present cytomegalovirus in arterial walls of patients suffering from grade III atherosclerosis
Chapter 5	Cytomegalovirus nucleic acid distribution within the human vascular tree
Chapter 6	Widespread presence of cytomegalovirus DNA in tissues of previously healthy trauma victims
Chapter 7	Detection and sequence analysis of the major immediate early and PP150 gene of latent human cytomegalovirus in spleen, liver and kidney tissues of trauma victims
Chapter 8	General discussion
	Samenvatting
	Curriculum vitae
	Nawoord

List of Abbreviations

BH-probe	BamH1-HindIII-probe
BSA	Bovine serum albumin
CPE	Cytopathologic effect
CMV	Cytomegalovirus
ES-fragment	EcoR1-Sall-fragment
E-genes	Early-genes
ES-probe	EcoR1-Sall-probe
HCMV	Human cytomegalovirus
HEF	Human embryonic fibroblasts
HGE	Human genomic equivalents
HSV	Herpes simplex virus
H&E	Hemotoxylin and eosin
IEA	Immediate early antigen
L-genes	Late genes
McAB's	Monoclonal antibodies
MCMV	Mouse cytomegalovirus
MDV	Marek's disease virus
PBS	Phospate-buffered-saline
PCR	Polymerase chain reaction
RCMV	Rat cytomegalovirus
VGE	Viral genomic equivalents

CHAPTER 1

Introduction. Aims of the study

1. Introduction
2. Aims of the study
3. Outline of the study
4. References

1. Introduction

Infections with human cytomegalovirus (HCMV) are widespread and the prevalence of these infections based on serological studies ranges from 40 to 90% (1). In immunocompetent individuals primary HCMV infection usually passes unnoticed and only a minority of individuals will develop a mononucleosis-like syndrome (2, 3). Primary infection can be followed by a prolonged period of active HCMV replication characterized by virus shedding from many body sites. This period is defined as persistent infection (4). Also recurrent infections defined as intermittent periods of viral shedding have frequently been observed (5, 6). The observation that HCMV can be transmitted by bloodtransfusions (7, 8) and organ transplants (9, 10) obtained from healthy individuals in whom viral shedding was not observed (11, 12) lead to the conclusion that a HCMV infection can become latent. This latent state can be defined as an infection in which the viral genome is present but no infectious virus is produced (13, 14, 15). Reactivation from this latent state into an active infection leading to endogenous reinfection can cause serious disease in severe immunocompromised individuals like transplant recipients and AIDS patients (1).

The question which cells harbor the latent virus has not been clearly answered yet. Earlier studies suggested that circulating lymphocytes contained latent HCMV (16) and in another study it was suggested that mononuclear cells in the kidney contained latent HCMV (17). Later on HCMV DNA was detected in vascular smooth muscle cells (18, 19, 20, 21) and a role for this virus in the pathogenesis of atherosclerosis was postulated. Also HCMV immediate early antigens but not late antigens could be detected in cells located in different organs such as liver, spleen, heart, kidney, and adrenals (22). Circulating blood mononuclear cells obtained from healthy blood donors were also demonstrated to contain HCMV DNA (23, 24, 25, 26, 27), however, other investigators failed to demonstrate the presence of HCMV DNA in peripheral leucocytes (28, 29). Also in bone-marrow cells obtained from healthy individuals HCMV nucleic acids could be detected, and recently two novel classes of HCMV latency associated transcripts have been described in these cells (30, 31).

2. Aims of the study

The aims of the present study were:

1. To develop methods suitable for the detection of HCMV nucleic acids in human tissues.
2. To study the presence of HCMV in the human arterial wall.
3. To investigate other human tissues obtained from healthy subjects for the presence of HCMV and characterise the cell type(s) in which the virus is present.

3. Outline of the study

The outline of the study is as follows.

In chapter 2 a review is presented of current literature concerning HCMV infection with special interest on the data related to epidemiology, tissue tropism during active infection and during latency.

In chapter 3 and 4 the presence of HCMV in arteries obtained from atherosclerotic and non-atherosclerotic patients is investigated with special emphasis on HCMV DNA, viral antigens and infectious virus.

In chapter 5 the findings of chapters 3 and 4 are extended

to other arteries not prone for the development of atherosclerosis and an estimation of

the HCMV viral load in the arterial wall is presented.

In chapter 6 the detection of HCMV DNA in a wide variety of human tissues obtained from previously healthy individuals is presented.

In chapter 7 the presence of HCMV DNA in spleen, kidney, and liver specimen obtained from previously healthy trauma victims is described and sequence analysis data are presented indicating that indeed HCMV is detected and that probably during latency more than one latent HCMV genotype can be present within a single individual.

All our findings are discussed in chapter 9.

In chapter 10 the main conclusions of this study are summarized

4. References

1. Ho M. Cytomegalovirus biology and infection. New York; Plenum Medical Book Co. 1991.
2. Jordan MC, Rousseau WE, Steward JA, Noble GR, Chin TDY. Spontaneous cytomegalovirus mononucleosis: clinical and laboratory observations in nine cases. *Ann.Intern.Med.* 1973; 97:153-160.
3. Horwitz CA, Henle W, Henle G. Diagnostic aspects of the cytomegalovirus mononucleosis syndrome in previously healthy persons. *Postgrad.Med.* 1979; 66:153-158.
4. Klemola E, von Essen R, Wager O, Haltia K, Koivuniemi A, Salmi I. Cytomegalovirus mononucleosis in previously healthy individuals. *Ann.Intern.Med.* 1969; 71:11-19.
5. Reynolds DW, Stagno S, Hosty TS, Tiller m, Alford JA Jr. Maternal cytomegalovirus excretion and perinatal infection. *N.Engl.J.Med.* 1973; 289:1-5.
6. Stagno S, Reynolds DW, Tsiantos a, et al. Cervical cytomegalovirus excretion in pregnant and nonpregnant women: suppression in early gestation. *J.Infect.Dis.* 1975; 133:522-7.
7. Prince AM, Szmunes W, Millian SJ. A serologic study of cytomegalovirus infections associated with blood transfusions. *N.Engl.J.Med.* 1971; 284:1125-1131.
8. Adler SP. New insights into human cytomegalovirus infections. *Prog.Med.Virol.* 1990; 37:136-155.
9. Ho M, Suwansirikul S, Dowling JN, Youngblood LA, Armstrong JA. The transplanted kidney as a source of cytomegalovirus infection *N.Engl.J.Med.* 1975; 293:1-109-1112.
10. Betts RF, Freeman RB, Douglas RG Jr, Talley TE, Rundell B. Transmission of cytomegalovirus infection with renal allograft. *Kidney Int.* 1975; 8:385-392.
11. Chou S. Acquisition of donor strains of cytomegalovirus by renal-transplant recipients. *N.Engl.J.Med.* 1986; 314:1418-1422.
12. Chou S. Cytomegalovirus infection and reinfection transplanted by heart transplantation. *J.Infect.Dis.* 1987; 155:1054-1056.
13. Stevens JG. Human herpesviruses: a consideration of the latent state. *Microbiol.Rev.* 1989; 53:318-332.
14. Banks AB, Rouse BT. Herpesviruses - immune escape artists? *Clin.Infect.Dis.* 1992; 14:933-941.
15. Fraser NW, Block TM, Spivack JG. The latency-associated transcripts of herpes simplex virus: RNA in research and function. *Virol.* 1992; 191:1-8.
16. Schrier RD, Nelson JA, Oldstone BA. detection of human cytomegalovirus in peripheral blood lymphocytes in a natural infection. *Science* 1985; 230:148-151.
17. Gnann JW, Ahlmen J, Svalander C, Olding L, Oldstone MBA, Nelson JA. Inflammatory cells in transplanted kidneys are infected by human cytomegalovirus. *Am.J.Pathol.* 1988; 132:239-248.

18. Petri BL, Melnick JL, Adam E, Burek J, McCollum CH, DeBaakey ME. Nucleic acid sequences of cytomegalovirus in cells cultured from human arterial tissue. *J.Infect.Dis.* 1987; 155:158-159.
19. Yamashiroya HM, Ghosh L, Yang R, Robertson AL. Herpes viridae in the coronary arteries and aorta of young trauma victims. *Am.J.Pathol.* 1988; 130:71-79.
20. Tanaka S, Toh Y, Komori K, Okadoma K, Sugimachi K. Possible role of cytomegalovirus in the pathogenesis of inflammatory aortic disease: a preliminary report. *J.Vasc.Surg.* 1992; 16:274-279.
21. Melnick JL, Hu C, Burek J, Adam E, Debaakey ME. Cytomegalovirus DNA in arterial walls of patients with atherosclerosis. *J.Med.Virol.* 1994; 42:170-174.
22. Toorkey CB, Carrigan DR. Immunohistochemical detection of an immediate early antigen of human cytomegalovirus in normal tissues. *J.Infect.Dis.* 1989; 160:741-751.
23. Stanier P, Taylor DL, Kitchen AD, Wales N, Tryhorn Y, Tyms AS. Persistence of cytomegalovirus in mononuclear cells in peripheral blood from blood donors. *Br.Med.J.* 1989; 299:897-898.
24. Bevan IS, Daw RA, Day PJ, Ala FA, Walker MR. Polymerase chain reaction for the detection of human cytomegalovirus infection in a blood donor population. *Br.J.Haematol.* 1991; 78:94-99.
25. Taylor-Wiedeman J, Sissons JGP, Borysiewics LK, Sinclair JH. Monocytes are a major site of persistence of human cytomegalovirus in peripheral blood mononuclear cells. *J.Gen.Virol.* 1991; 72:2059-2064.
26. Taylor-Wiedeman J, Hayhurst GP, Sissons JGP, Sinclair JH. Polymorphonuclear cells are not sites of persistence of human cytomegalovirus in healthy individuals. *J.Gen.Virol.* 1993; 74:265-268.
27. Zhang LJ, Hanff P, Rutherford C, Churchill WH, Crumpacker CS. Detection of human cytomegalovirus DNA, RNA, and antibody in normal donor blood. *J.I.D.* 1995; 171:1002-6.
28. Bitch A, Kirchner H, Dupke R, Bein G. Failure to detect human cytomegalovirus DNA in peripheral blood leucocytes of healthy blood donors by the polymerase chain reaction. *Transfusion* 1992; 32: 612-617.
29. Urushibara N, Kwon KW, Takahashi TA, Sekiguchi S. Human cytomegalovirus DNA is not detectable with nested double polymerase chain reaction in healthy blood donors. *Vox Sang* 1995; 68:9-14.
30. Kondo K, Mocarsky ES. Cytomegalovirus latency and latency-specific transcription in hematopoietic progenitors. *Scand.J.Infect.Dis. Suppl.* 1995; 99:63-67.
31. Kondo K, Xu J, Mocarski ES. Human cytomegalovirus latent gene expression in granulocyte-macrophage progenitors in culture and in seropositive individuals. *Proc. Natl. Acad. Sci. USA* 1996; 93:11137-11142.

CHAPTER 2

General introduction

- 2.1. Introduction
- 2.2. Epidemiology of HCMV infections
 - 2.2.1. Epidemiology of HCMV in the general population
 - 2.2.2. Epidemiology of HCMV in the immunocompromised host
- 2.3. HCMV gene expression
 - 2.3.1. In vitro HCMV infection of permissive cells
 - 2.3.2. In vitro HCMV infection of non permissive cells
 - 2.3.3. In vitro HCMV latency models
- 2.4. Distribution of HCMV in the human body
 - 2.4.1. Tissue distribution during active HCMV disease
 - 2.4.2. Tissue distribution of HCMV in non HCMV related disease
 - 2.4.3. Tissue distribution of HCMV in healthy subjects
- 2.5. Animal models for CMV latency
- 2.6. References

2.1. Introduction

Infections with human cytomegalovirus (HCMV) are common and the prevalence of these infections as based on serological studies ranges from 40-90% (1). In the immunocompetent individual, clinical disease is a relatively exceptional event (1). In immunocompromised patients, like transplant recipients (2) and AIDS patients (3), HCMV can cause diseases as interstitial pneumonitis and retinitis. In solid organ transplant recipients, the infection mostly occurs by endogenous reinfection due to reactivation of latent virus present in the donor organ but also reactivation of latent virus of the recipient is possible (4, 5, 6, 7). In AIDS patients and in allogeneic bone marrow transplant recipients, most infections result from reactivation of latent virus already present in the host (8). The exact nature of the cells from which the latent HCMV reactivates, however, remains elusive. Some investigators suggest that peripheral leucocytes (9, 10, 11), vascular smooth muscle cells (12, 13, 14), and various celltypes in spleen, liver, kidney, heart and adrenals (15, 16, 17) contain HCMV DNA during latency.

The exact localisation of latent HCMV is still not clearly defined. In this chapter, a brief review of the literature relevant to the understanding of HCMV latency is presented.

2.2. Epidemiology of HCMV infections

As suggested by Ho (18), epidemiology of HCMV infections can be divided in two fields. One field describing the normal patterns of spread in the general population, and a second field describing the HCMV spread in a non-immunocompetent population. From both fields much can be learned about the complex biology of HCMV.

2.2.1. Epidemiology of HCMV in the general population

Infections with HCMV have a history in science of more than 9 decades. The typical inclusion bodies detected in organs of newborns were first discovered in 1904 when Ribbert (19) described "protozoan-like" cells in the kidney of a stillborn. The presence of these typical inclusion bodies in the organs of newborns was confirmed by several other authors and the disorder was named cytomegalic inclusion disease (CID). The causative agent however remained unknown and only newborns were considered to be vulnerable. In 1932, Farber and Wolbach (20) demonstrated the presence of inclusion bodies in the submaxillary glands but also in other organs of 12% of infants dying of various diseases. These findings showed that CID was not limited to newborns and that the prevalence was probably much higher than presumed. During the late fifties it was demonstrated that the causative agent of CID was an infectious non-bacterial micro-organism (21, 22, 23) and in 1960 the micro-organism causing cytomegalic inclusion disease was identified, and termed Human Cytomegalovirus (HCMV) (24). In 1965 it was shown by Klemola (25) that in healthy individuals HCMV infection can lead to a mononucleosis-like disease. The characteristics of this disease closely resembled the clinical symptoms occurring in infectious mononucleosis caused by Epstein Barr virus where atypical lymphocytes are also observed. Until now the mononucleosis-like syndrome is the only disease occurring in the normal population where a causative role for HCMV is firmly established. Other infrequent diseases caused by HCMV are: hepatitis, the Guillain-Barre' syndrome, meningoencephalitis, myocarditis, and thrombocytopenia-hemolytic anemia (1). Recently, much is speculated on the role of HCMV in the development of atherosclerosis (26, 27, 28).

During infection, HCMV induces a humoral and cellular immune response. Due to the development of new serological techniques in the 1960's, sensitive methods became

available for the sero-epidemiological study of HCMV. Using these methods it was shown that the prevalence of HCMV antibodies was high, ranging from 40 to 100% depending largely on the socio-economic status (29). Analysis of age-related prevalence showed two periods of increased infection rates during life. The first was the perinatal period, and the second occurs during the years of sexual maturity. In the perinatal period transmission can occur vertically from mother to child: at birth by passing a contaminated uterine cervix (30) and later by breast-feeding (31, 32). Horizontal transmission can also occur, especially in day care centers, from other infected children (33). During the years of sexual maturity transmission occurs during heterosexual and homosexual intercourse. Both the uterine cervix and the semen of healthy adults can contain HCMV (34, 35). In some cases HCMV strains isolated from sexual partners had identical DNA restriction fingerprinting patterns proving this mode of transmission (36).

2.2.2. Epidemiology of HCMV in the immunocompromised host

In solid organ transplantations, HCMV infection occurs in 50%-100% of the recipients (1, 2, 4, 18, 47, 48, 49, 50). These infections can be divided in three groups. 1) primary infection of a seronegative recipient caused by HCMV present in the donor organ. 2) reactivation of latent HCMV in the seropositive recipient and 3) reinfection of a seropositive recipient by HCMV present in the donor organ. The clinical manifestations of these HCMV infections range from subclinical to often fatal disease (1, 18) and are related to the type of transplantation and the degree of immunosuppression used. In general, primary infections tend to be more severe in these patients than in immunocompetent individuals (2, 49, 51).

In bone marrow transplant recipients, the major risk factor for severe HCMV disease is HCMV seropositivity (1, 8, 52). In these patients HCMV infection is caused by reactivation of latent virus.

HCMV can also be transmitted by transfusion of blood and/or blood products (53). The strict exclusion of blood obtained from seropositive donors indeed decreased the risk of HCMV transmission (54). Also in neonates HCMV can be transmitted by bloodtransfusions (55, 56). There have been numerous efforts to isolate HCMV from human blood, but active virus could never be detected (1), suggesting that the virus is present in a latent, non-replicating state.

From the epidemiology of HCMV it can be concluded that after primary infection, HCMV probably develops a lifelong relationship with its host, a status called latent infection or latency (57, 58).

During the last decades several definitions have come into use to describe the complex biological behaviour of HCMV. It therefore is necessary to discuss some of them. Primary infection is the infection obtained for the first time in life (i.e. in a seronegative individual). It results in an active infection meaning that there is viral replication in various cell types and in several organs. HCMV disease however is very rare (1, 15, 17, 20). In some subjects primary infection can be followed by a prolonged period of HCMV replication defined as a persistent infection (59). Also intermittent periods of viral shedding defined as recurrent infections have frequently been observed (30, 60). Later on, no infectious HCMV can be detected anymore, so the virus has become latent (61). A definition of this latent state is proposed by Banks and Rouse (62) as a persistent infection in which the viral genome is present, but gene expression is absent or limited and infectious virus is not produced. Later Fraser (63) suggested that the capacity to reactivate from this latent state into an active infection should be included in this definition. Reactivation from this latent state into active infection occurs in the immuno-

compromised host but perhaps also in the "normal" i.e. immunocompetent host, as has been known for other herpesviruses such as human simplex virus which can reactivate after stimuli as UV-irradiation, fever, and even stress (64).

2.3. HCMV gene expression

HCMV, a member of the herpesviridae, is a large, enveloped, doublestranded DNA virus (65) with a genome consisting of 235 kilo basepairs containing 208 predicted open reading frames probably encoding more than 200 polypeptides (66). The functions of many of these polypeptides are not well understood. Some are regulatory proteins, like the immediate early gene products IE-1 and IE-2 (67). Both proteins control viral replication (65, 68, 69). Other proteins make up the infectious viral particle (65, 70). HCMV has a restricted host-range and in vitro only human fibroblasts (65), vascular smooth muscle cells (71), differentiated macrophages (72) and some cell lines after induction of differentiation (73, 74, 75) are permissive. Productive replication is not observed in other cell types and viral expression is often limited to the immediate-early genes (65, 67, 68).

2.3.1. in vitro HCMV infection of permissive cells

Attachment to and penetration of the cell by fusion of the viral envelope and the plasma membrane (76) is completed within 5 minutes (77). Thereafter HCMV is transported to the nucleus of the cell and within 30 minutes viral gene products can be detected (78). In the nucleus of the cell the viral DNA is transcribed in a stepwise fashion (79, 80). This replication cycle is marked by three distinct phases: immediate-early (IE), early (E), and late (L) based on the time of synthesis after entry of HCMV into the cell.

The IE-genes are transcribed immediately after viral entry into the cell. Proteins coded for by these genes can be detected using monoclonal antibodies as soon as 30 minutes after infection (78, 80). The gene segment coding for the most abundantly transcribed IE-proteins is located in the unique long segment of HCMV (81, 82, 83). Besides the major IE-genes (UL122, UL123) there are three other regions of IE transcription (US3, UL36, UL37) (79). The relative amount of RNA transcribed from these regions is low. Initial transcription of IE-genes takes place in the absence of viral protein synthesis by cellular polymerases, probably RNA polymerase II (83). Two IE-gene products, IE-1 (UL123) and IE-2 (UL122) are abundantly translated. The role of the IE-1 protein appears to be autoregulatory in activating HCMV IE-sequences but probably also in activating cellular genes. The IE-2 protein plays a central role in the switch from IE- to E-gene expression (84, 86). Part of the IE-2 gene is also abundantly transcribed during L-gene expression and functions as a repressor of IE-gene expression during this period (86, 87). The reason for this is not yet clear. After transactivation of E-genes by IE-2 proteins these genes remain active until the late transcription period. E-genes are encoded from a large proportion of the viral genome but the role of these genes, besides transactivation of L-genes, is poorly understood (67, 79). During early times of infection, proteins needed for viral DNA replication are produced (67, 79). Proteins encoded by L-genes are mainly translated after viral DNA synthesis has started, although they can be transcribed at early times of infection (67, 79). Products of both E-genes and L-genes are assembled into infectious HCMV particles, and are called "structural proteins" (70). Viral assembly mainly occurs in the cell nucleus where DNA is packaged into capsids. The envelope of the progeny virions is acquired from the nuclear membrane (79, 88, 89). From there on the viral particles are transported via the Golgi apparatus to the cell surface and are probably released from these cells by an exocytic

pathway (79, 90).

2.3.2. in vitro HCMV infection of non-permissive cells

In vitro it seems that mainly human fibroblasts fully support HCMV replication. In vitro infection of other celltypes only supports limited viral replication (71, 72, 73, 74, 75), or no production of progeny virus at all (79, 91). The block to HCMV replication in nonpermissive cells occurs at a step prior to viral DNA synthesis and is restricted to expression of IE- and sometimes to E-genes (92, 93, 94, 95). The nature of the block suppressing further viral transcription in these nonpermissive cell types is not clear. Some cell types like monocytes and unstimulated macrophages which are nonpermissive in vitro can support HCMV replication after induction of differentiation (96, 97). However, the mechanism responsible for this switch also remains unclear.

2.3.3. in vitro HCMV latency models

Several attempts have been made to establish an in vitro latency model for HCMV. Firstly by in vitro induction of differentiation of nonpermissive cell types as macrophages (96, 97) and human embryonal carcinoma cells (74). Secondly by infecting and incubating human thyroid papillary carcinoma cells (TPC-1) at 40.5°C with HCMV. Under these conditions the cultures could be maintained for 65 days without detection of infectious virus. However, by decreasing the culture temperature to 37°C infectious virus could be detected (98). Thirdly by treating fibroblasts with aciclovir and interferon-gamma and maintaining cultures at 40.5°C (99). Under these conditions viral replication could be stopped for more than 70 days and returning to normal culture conditions did restore HCMV replication. Most of these models demonstrated a restricted transcription of IE- and sometimes E- genes, but never of L-genes during the presumed latent state. Recently two novel classes of HCMV latency associated transcripts have been detected in bone marrow aspirates from healthy HCMV seropositive individuals (100, 101). Both transcripts are complementary to the IE-1 and IE-2 gene regions. One class, sense transcripts, is encoded in the same direction as productive phase transcripts and encodes a 94 aa protein. The other class, antisense transcripts, is complementary to the IE-1 gene exons 2-4 and possibly encode for a 152 and a 154 aa protein. Antibody to these 94 and 154 aa proteins is detectable in the serum of healthy HCMV seropositive individuals. The role of these proteins in the establishment of a latent infection remains unknown. These findings could not be reproduced by others (102, 103) who also detected HCMV genomes in bone marrow cells, but were unable to detect HCMV lytic gene expression (i.e. IE-1 and IE-2 sense transcripts).

From these in vitro data it could be concluded that after penetration of HCMV into a cell transcription of immediate early genes starts and, depending on the cell type, transactivation of E- and L- genes will or will not follow. Based on these in vitro models it is possible that after HCMV infection IE-genes are transcribed in most cell types and probably these genes are also transcribed during latency.

2.4. distribution of HCMV in the human body

HCMV antigens and/or nucleic acids have recently been detected in various samples obtained from patients with or without active HCMV infection. Furthermore HCMV DNA and/or antigens have been detected in blood obtained from healthy blood donors. From

these data it can be concluded that HCMV shows a wide tissue distribution not only during active infection, but probably also during latency.

2.4.1. tissue distribution of HCMV during active infection

As has been described in previous sections, severe HCMV infections occur in immunocompromised patients. In this group of patients most studies on the tissue distribution of active HCMV infection are performed. By in situ DNA hybridization (ISH), HCMV nucleic acids could be demonstrated in lung, pharynx, lymph node, adrenal, liver, kidney, small intestine and salivary gland samples obtained from AIDS patients with clinical active HCMV disease (104). Also in solid organ transplantation recipients, HCMV nucleic acids and antigens could be detected by ISH in various organs (1, 16, 105, 106, 107, 108). Based on histopathological findings, mostly epithelial cells seemed targets for viral replication (1), but by using ISH also a variety of other cell types including fibroblasts, and endothelial cells were found to contain viral nucleic acids (105, 106, 107, 108). During active HCMV infection the virus can be readily detected in blood leucocytes using tissue culture techniques, ISH, and polymerase chain reaction (PCR) (1, 15, 109, 110, 111). HCMV could not only be detected in monocytes and granulocytes, but also in CD4+, CD8+ and CD19+ lymphocytes (111).

2.4.2. Tissue distribution in potentially HCMV related disease

The relationship between HCMV and disease has been firmly established by using histopathological techniques (see section 2.2.1). Later on HCMV has been associated with several "new" diseases primarily based on the detection of HCMV DNA and/or RNA in lesions typical for the disease under investigation. Already in 1981, HCMV DNA was detected in Kaposi's sarcoma and a role for this virus in the pathogenesis of this disease was suggested (112). Later it became obvious that not HCMV but another herpesvirus (human herpesvirus 8) played a causative role (113, 114). HCMV DNA was also detected in human tonsillar lymphocytes (115), the human cervix (116), lymph nodes of patients with angioimmunoblastic lymphadenopathy (117), and in various other tissues (1). Based on the observation that an avian herpesvirus named Marek's disease virus (MDV) can cause atherosclerosis in chickens (118), a role for HCMV in the initial events leading to human atherosclerosis was suggested (26, 28, 119, 120). Indeed HCMV DNA could be detected in samples of carotid arteries and abdominal aortas obtained during vascular reconstructive surgery (12, 121, 122), but also in samples of coronary arteries and abdominal aortas of young trauma victims with minimal signs of atherosclerotic disease (23).

The question now is whether there is a causal relationship between the presence of HCMV nucleic acids in the lesions and the development of the disease. A possible mechanism might be inhibition of the tumor suppression protein p53 by HCMV proteins as has been demonstrated in patients who develop restenosis after coronary angioplasty (123).

2.4.3. Tissue distribution of HCMV in previously healthy subjects

To date, only a limited number of studies have been performed to detect HCMV in tissue samples obtained at autopsy from previously healthy individuals. In one study (17) HCMV immediate early antigens could be demonstrated with monoclonal antibodies

in kidney, spleen, liver, lung, and brain tissues derived from HCMV-seropositive healthy individuals. In another study, HCMV nucleic acids could be detected by DNA hybridization techniques with a DNA probe reactive with immediate early sequences in splenic tissues of organ donors without clinical or laboratory confirmed active HCMV infection (124). Also bone marrow progenitor cells from healthy individuals can contain HCMV DNA as demonstrated by polymerase chain reaction (PCR) (125). More data are available on the presence of HCMV DNA in leucocytes obtained from healthy blood donors. According to several reports, 40-80% of blood donors have detectable HCMV DNA mainly in blood mononuclear cells (9, 10, 11, 126, 127). These data, however, are not conclusive since other investigators failed to detect HCMV DNA in leucocytes of healthy blood donors (128, 129). Most reports only use a PCR primer set reactive with the major immediate early gene (mostly UL123) (9, 11, 126, 128, 129) to detect HCMV DNA. Since immediate early sequences are highly conserved among the herpesviridae it is possible, albeit unlikely, that another herpesvirus was detected. Only in the report of Bevan et al. (10) three different primer sets reactive with HCMV DNA derived from the immediate early region (UL123), the HXLF6 region or the GP64 region (UL83) were used. In this study all HCMV-PCR positive blood samples were simultaneously positive with all three primer sets suggesting that indeed the whole viral genome is present.

2.5. Animal models for HCMV latency

There are several animal models for CMV (130, 131) but in only two, the murine CMV model and the rat CMV model, data are available concerning CMV latency (58). The murine cytomegalovirus (MCMV) is extensively investigated. During active infection in mice MCMV closely resembles HCMV with respect to tissue distribution and many organs support viral replication (127). MCMV can be detected in blood mononuclear cells (132, 133, 134), the liver (135), the spleen (136) and the skin (137). After entry into the cell MCMV is also transcribed in a stepwise fashion (138). MCMV readily establishes a latent infection (139) and during latency viral genomes can be detected in a variety of organs like brain, heart, kidney liver, lung, spleen, and salivary gland, but not in leucocytes as demonstrated by PCR and RT-PCR (140, 141, 142, 143). It also has been demonstrated that immediate early genes are transcribed in spleens of latently infected mice (144). The rat cytomegalovirus (RCMV) on the other hand is a more convenient model for transplantation experiments. The RCMV also closely resembles the HCMV in both infection pattern (145) and molecular biology (146, 147, 148). It also establishes a latent infection with can be transmitted to a suitable host by transplantation (149, 150). The virus also reactivates under conditions of immunosuppression (151). Until now no data are available on the localization of latent RCMV nor on the transcriptional program during latency.

2.6. References:

1. Ho M. Cytomegalovirus biology and infection. New York; Plenum Medical Book Co. 1991.
2. Rubin RH. Impact of cytomegalovirus infection on organ transplant recipients. *Rev.Infect.Dis.* 1990; 12:S754-S766.
3. Schooley RT. Cytomegalovirus in the setting of infection with human immunodeficiency virus (see comments). *Rev.Infect.Dis.* 1990; 12:S811-S819.
4. Ho M, Suwansirikul S, Dowling JN, Youngblood LA, Armstrong JA. The transplanted kidney as a source of cytomegalovirus infection *N.Engl.J.Med.* 1975; 293:1-109-1112.
5. Betts RF, Freeman RB, Douglas RG Jr, Talley TE, Rundell B. Transmission of

- cytomegalovirus infection with renal allograft. *Kidney Int.* 1975; 8:385-392.
6. Chou S. Acquisition of donor strains of cytomegalovirus by renal-transplant recipients. *N.Engl.J.Med.* 1986; 314:1418-1422.
7. Chou S. Cytomegalovirus infection and reinfection transplanted by heart transplantation. *J.Infect.Dis.* 1987; 155:1054-1056.
8. Rubie H, Attal M, Campardou AM, Gayet-Mengelle C, Payen C, Sanguignol F, Calot JP, Charlet JP, Robert A, Huguet F, Puel J, Pris J, Laurent G. Risk factors for cytomegalovirus infection in BMT recipients transfused with seronegative blood products. *Bone Mar.Transpl.* 1993; ??;209-214.
9. Stanier P, Taylor DL, Kitchen AD, Wales N, Tryhorn Y, Tyms AS. Persistence of cytomegalovirus in mononuclear cells in peripheral blood from blood donors. *Br.Med.J.* 1989; 299:897-898.
10. Bevan IS, Daw RA, Day PJ, Ala FA, Walker MR. Polymerase chain reaction for the detection of human cytomegalovirus infection in a blood donor population *Br.J.Haematol.* 1991; 78:94-99.
11. Taylor-Wiedeman J, Sissons JGP, Borysiewics LK, Sinclair JH. Monocytes are a major site of persistence of human cytomegalovirus in peripheral blood mononuclear cells. *J.Gen.Virol.* 1991; 72: 2059-2064.
12. Petri BL, Melnick JL, Adam E, Burek J, McCollum CH, DeBaKey ME. Nucleic acid sequences of cytomegalovirus in cells cultured from human arterial tissue. *J.Infect.Dis.* 1987; 155:158-159.
13. Yamashiroya HM, Ghosh L, Yang R, Robertson AL. Herpes viridae in the coronary arteries and aorta of young trauma victims. *Am.J.Pathol.* 1988; 130:71-79.
14. Tanaka S, Toh Y, Komori K, Okadoma K, Sugimachi K. Possible role of cytomegalovirus in the pathogenesis of inflammatory aortic disease: a preliminary report. *J.Vasc.Surg.* 1992; 16:274-279.
15. Schrier RD, Nelson JA, Oldstone BA. detection of human cytomegalovirus in peripheral blood lymphocytes in a natural infection. *Science* 1985; 230:148-151.
16. Gnann JW, Ahlmen J, Svalander C, Olding L, Oldstone MBA, Nelson JA. Inflammatory cells in transplanted kidneys are infected by human cytomegalovirus. *Am.J.Pathol.* 1988; 132:239-248.
17. Toorkey CB, Carrigan DR. Immunohistochemical detection of an immediate early antigen of human cytomegalovirus in normal tissues. *J.Infect.Dis.* 1989; 160:741-751.
18. Ho M. Epidemiology of cytomegalovirus infections. *Rev.Infect.Diseas.* 1990; 12 (suppl.7):S701-S710.
19. Ribbert. Ueber protozoenartige zellen in der niere eines syphilitischen neugeborenen und in der parotis von kindern. *Centralbl.Allgem.Pathol.u.Pathol.Anat.* 1904; 23:945-948.
20. Farber S, Wolbach SB. Intranuclear and cytoplasmic inclusions (protozoanlike bodies) in the salivary glands and other organs of infants. *Am.J.Pathol.* 1932; 8:123-35.
21. Rowe WP, Hartley JW, Waterman S, Turner HC, Huebner RJ. Cytopathogenic agent resembling human salivary gland virus recovered from tissue cultures of human adenoids. *Proc.Soc.Exp.Biol.Med.* 1956; 92:418-424.
22. Weller TH, Macauley IC, Graig JM, Wirth P. Isolation of intranuclear inclusion producing agent from infants with illnesses resembling cytomegalic inclusion disease. *Proc.Soc.Exp.Biol.Med.* 1957; 94:4-12.
23. Smith MG. Propagation in tissue cultures of a cytopathic virus from human salivary gland virus (SGV) disease. *Proc.Soc.Exp.Biol.Med.* 1956; 92:424-430.
24. Weller TH, Hanshaw JB, Scott DE. Serologic differentiation of virusses responsible

- for cytomegalic inclusion disease. *Viol.* 1960; 130-132.
25. Klemola E, Kaariainen L. Cytomegalovirus as a possible cause of a disease resembling infectious mononucleosis. *Br.Med.J.* 1965; 1:1099-1102.
26. Hajjar DP. Viral pathogenesis of atherosclerosis. *A.J.P.* 1991; 139:1195-1211.
27. Consigny PM. Pathogenesis of atherosclerosis. *A.J.R.* 1995; 164:553-558.
28. Melnick JL, Adam E, DeBakey ME. Possible role of cytomegalovirus in atherogenesis. *J.A.M.A.* 1990; 263:2204-2207.
29. Yow MD, Williamson DW, Leeds LJ, Thompson P, Woodward RM, Walms BF, Lester JW, Six HR, Griffiths PD. Epidemiological characteristics of cytomegalovirus infection in mothers and their infants. *Am.J.Obstet.Gynecol.* 1988; 158:1189-1195.
30. Reynolds DW, Stagno S, Hosty TS, Alford CA Jr.. Maternal cytomegalovirus excretion and perinatal infection. *N.Engl.J.Med.* 1973; 289:1-5.
31. Stagno S, Reynolds DW, Pass RF, Alford CA. Breast milk and the risk of cytomegalovirus infection *N.Engl.J.Med.* 1980; 302:1073-1074.
32. Hayes K, Danks DM, Gibas H. Cytomegalovirus in human milk. *N.Engl.J.Med.* 1972; 287:177-178.
33. Pass RF, August AM, Dworsky M. Cytomegalovirus infection in a day care center. *N.Engl.J.Med.* 1982; 307:477-479.
34. Chandler SH, Handsfield HH, McDougall JK. Isolation of multiple strains of cytomegalovirus from women attending a clinic for sexually transmitted diseases. *J.Infect.Dis.* 1987; 155:655-660.
35. Lang DJ, Kummer JF. Demonstration of cytomegalovirus in semen. *N.Engl.J.Med.* 1972; 287:756-758.
36. Handsfield HH, Chandler SH, Caine VA, Meyers JD, Corey L, Medeiros E, McDougall JK. Cytomegalovirus infection in sex partners: evidence for sexual transmission. *J.Infect.Dis.* 1985; 151:344-348.
37. Marker SC, Howard RJ, Simmons RL, et al Cytomegalovirus infection: a quantitative prospective study of 320 consecutive renal transplants. *Surgery* 1981; 89:660-671.
38. Peterson PK, Balfour HH Jr, Marker SC, Fryd DS, Howard RJ, Simmons RL. Cytomegalovirus disease in renal allograft recipients: a prospective study of the clinical features, risk factors and impact on renal transplantation. *Medicine* 1980; 59:283-300.
39. Rand KH, Pollard RB, Merigan TC. Increased pulmonary superinfections in cardiac transplant patients undergoing primary cytomegalovirus infection. *N.Engl.J.Med.* 1978; 298:951-3.
40. Aldrete JS, Sterlij WA, Hathaway BM, Morgan JM, Diethelm AG. Gastrointestinal and hepatic complications affecting patients with renal allografts. *Am.J.Surg.* 1975; 129:115-24.
41. Anuras S, Piros J, Bonney WW, Forker EL, colville DS, Corry RJ. Live disease in renal transplant recipients *Arch.Intern.Med.* 1977; 137:42-8.
42. Luby JP, Brunett W, Hull AR, Ware EJ, Shorey JW, Peters PC. Relationship between cytomegalovirus and hepatic function abnormalities in the period after renal transplantation. *J.infect.Dis.* 1974; 129:573-5.
43. Ware AJ, Luby JP, Hollinger B, et al. Etiology of liver disease in renal-transplant patients. *Ann.Intern.Med.* 1979; 91:364-71.
44. Egbert PR, Pollard RB, Gallagher JB, Merigan TC. Cytomegalovirus retinitis in immunosuppressed hosts. II. Ocular manifestations. *Ann.Intern.Med.* 1980; 93:664-70.
45. Jacobson MA, Mills J. Serious cytomegalovirus disease in the aquired immunodeficiency syndrome (AIDS). Clinical findings, diagnosis, and treatment. *Ann.In-tern.Med.* 1988; 108:585-94.

46. Pollard RB, Egbert PR, Gallagher JG, Merigan TC. Cytomegalovirus retinitis in immunosuppressed hosts. I Natural history and effects of treatment with adinine arabinoside. *Ann.Intern.Med* 1980; 93:655-64.
47. Fiala M, Paine JE, Berne TV, Moore TC, Henle W, Montgomerie JZ, Chatterjee SN, Guze LB. Epidemiologie of cytomegalovirus infection afetr transplantation and immunosuppression. *J.Infect.Dis.* 1975; 132:421-433.
48. Rubin RH, Tolkof-Rubin NE, Oliver D, Rota TR, Hamilton J, Betts RF, Pass RF, Hillis W, Szmunes W, Farrel ML, Hirsch MS. Multicenter seroepidemiologic study of the impact of cytomegalovirus infection on renal transplantation. *Transplantation* 1985; 40:243-249.
49. Ackerman JR, Lefor WM, Weinstein S, et al. Four-year experience with exclusive use of cytomegalovirus antibody (CMV-Ab)-negative donors for (CMV-Ab)-negative kidney recipients. *Transplant.Proc.* 1988; 20:469-71.
50. Chou SW, Norman DJ. The influence of donor factors other than serologic status on transmission of cytomegalovirus to transplant recipients. *Transplantation* 1988; 46:89-93.
51. Ho M. Observations from transplantation contributing to the understanding of pathogenesis of CMV infection. *Transpl.Proc.* 1991; 23 (suppl.3):104-109.
52. Winston DJ, Ho WG, Champlin RE. Cytomegalovirus infections after allogeneic bone marrow transplantation. *Rev.Infect.Dis.* 1990; 12 (suppl.7):S776-791.
53. Prince AM, Szuness W, Millian SJ. A serologic study of cytomegalovirus infections associated with blood transfusions. *N.Engl.J.Med.* 1971; 284:1125-1131.
54. Verdonck LF, de Haan-Hentzen YCE, Dekker AW, Mudde GC, de Gast GC. Cytomegalovirus seronegative platelets and leucocyte poor red blood cells from random donors can prevent primary cytomegalovirus infection after bone marrow transplantation. *Bone Marrow Transplant* 1987; 2:73-78.
55. Yeager AS, Grumet FC, Haffleigh EB, Bradley JS, Prober CG. Prevention of transfusion-acquired cytomegalovirus infection in newborn infants. *J.Pediatr.* 1981; 98:281-7.
56. Adler SP. Neonatal cytomegalovirus infections due to blood. *CRC Crit.Rev.Lab.-Sci.* 1984; 23:1-14.
57. Jordan MC. Latent infection and the elusive cytomegalovirus. *Rev.Infect.Dis.* 1983; 5:205-215.
58. Bruggeman CA. Cytomegalovirus and latency: an overview. *Virchows Archiv B Cell Pathol.* 1993; 64:325-333.
59. Klemola E, von Essen R, Wager O, Haltia K, Koivuniemi A, Salmi I. Cytomegalovirus mononucleosis in previously healthy individuals. *Ann.Intern.Med.* 1969; 71:11-19.
60. Stagno S, Reynolds DW, Tsiantos A, et al. Cervical cytomegalovirus excretion in pregnant and nonpregnant women: suppression in early gestation. *J.Infect.Dis.* 1975; 133:522-527.
61. Steven JG. Human Herpesviruses: a consideration of the latent state. *Microbiol.Rev.* 1989; 53:318-332.
62. Banks AB, Rouse BT, Herpesviruses-immune escape artists? *Clin.Infect.Dis.* 1992; 14:933-941.
63. Fraser NW, Block TM, Spivack JG. The latency-associated transcripts of herpes simplex virus: RNA in research and function. *Virol.* 1992; 191:1-8.
64. Whitley RJ, Gnann jr JW. The epidemiology and clinical manifestations of herpes simplex virus infections. In: Roizman B, Whitley RJ, Lopez C, ed. *The human herpesviruses*, New York, Raven press, LTD 1993.
65. Mocarski ES Jr. Cytomegaloviruses and their replication. In: Fields BN, ed. *Fields virology*, Philadelphia, lippincott-raven publishers, 1996.

66. Chee MS, Bankier AT, Beck S, Bohni R, Brown CM, Cerny R, Horsnell T, Hutchinson CA, Kouzarides T, Martignetti JA, Preddie E, Satchwell SC, Tomlinson P, Weston KM, Barrel BG. Analysis of the protein coding content of the sequence of human cytomegalovirus strain AD169. *Current topics in microbiology and immunology* 1990; 154:125-169.
67. Mocarski ES, Ahenes GB, Manning WC, Sambucetti LC, Cherrington JM. Molecular genetic analysis of cytomegalovirus gene regulation in growth, persistence, and latency. *Curr.Top.Microbiol.Immunol.* 1990; 154:47-74.
68. Nelson JA, Gnann JJ, Ghazal P. Regulation and tissue specific expression of human cytomegalovirus. *Curr.Top.Microbiol.Immunol.* 1990; 154:75-100.
69. Stenberg RM. Immediate-early genes of human cytomegalovirus: organization and function. In Huang ES, ed. *Molecular aspects of human cytomegalovirus diseases*. Heidelberg: Springer-Verlag, 1992
70. Spaete RR, Gehrz RC, Landini MP. Human cytomegalovirus structural proteins. *J.Gen.Virol.* 1994;75:3287-3308.
71. Tumilowicz JJ, Gawlik ME, Powell BB, Trentin JJ. Replication of cytomegalovirus in human arterial smooth muscle cells. *J.Virol.* 1985;56:839-845.
72. Ibanez CE, Schrier R, Ghazal P, Wiley C, Nelson JA. Human cytomegalovirus productively infects primary differentiated macrophages. *J.Virol.* 1991;65:6581-6588.
73. Dutko FJ, Oldstone MB. Cytomegalovirus causes a latent infection in undifferentiated cells and is activated by induction of cell differentiation. *J.Exp.Med.* 1981;1-54:1636-51.
74. Gonczol E, Andrew PW, Plotkin SA. Cytomegalovirus replicates in differentiated but not in undifferentiated human embryonal carcinoma cells. *Science* 1984;224:-159-61.
75. Shelbourn SL, Sissons JG, Sinclair JH. Expression of oncogenic ras in human teratocarcinoma cells induces partial differentiation and permissiveness for human cytomegalovirus infection. *J.Gen.Virol.* 1989;70:367-74.
76. Compton T, Nepomuceno RR, Nowlin DM. Human cytomegalovirus penetrates host cells by pH-independent fusion at the cell surface. *Virol.* 1992; 191:387-95.
77. Smith JD, de Harven E. Herpes simplex virus and human cytomegalovirus replication in WI-38 cells. II. An ultrastructural study of viral penetration. *J.Virol.* 1974; 14:945-56.
78. Michelson FS, Horodniceanu F, Guillon JC. Immediate early antigens in human cytomegalovirus infected cells. *Nature* 1977; 270:615-7.
79. Mocarski ES. Cytomegalovirus biology and replication. in: B. Roizman, R.J. Whitley, and C. Lopez, ed. *The human herpesviruses*, New York, Raven Press, Ltd., 1993: 173-226.
80. Otto SM, Sullivan TG, Malone CL, Stinski MF. Subcellular localization of the major immediate early protein (IE-1) of human cytomegalovirus at early times after infection. *Virol.* 1988; 162:478-482.
81. Stenberg RM, Thomson DR, Stinski MF. Structural analysis of the major immediate early gene of human cytomegalovirus. *J.Virol.* 1984; 49:190-9.
82. Stenberg RM, Witte PR, Stinski MF. Multiple spliced and unspliced transcripts from human cytomegalovirus immediate early region 2 and evidence for a common initiation site within immediate early region 1. *J.Virol.* 1985; 56:665-75.
83. Wilkinson GW, Akrigg A, Greenaway PJ. Transcription of the immediate early genes of human cytomegalovirus strain AD169. *Virus Res.* 1984; 1:101-6.
84. Thomson DR, Stenberg RM, Groins WF, Stinski MF. promotor-regulatory region of the immediate early gene of human cytomegalovirus. *Proc.Natl.Acad.Sci.USA* 1984; 81:659-63.
85. Chang CP, Malone CL, Stinski MF. A human cytomegalovirus early gene has

- three inducible promoters that are regulated differentially at various times after infection. *J.Virol.* 1989; 63:281-290.
86. Pizzorno MC, O'Hare P, Sha I, LaFemina RL, Hayward GS. Trans-activation and autoregulation of gene expression by the immediate-early region 2 gene products of human cytomegalovirus. *J.Virol.* 1988; 62:1167-1179.
 87. Pizzorno MC, Hayward GS. The ie2 gene products of human cytomegalovirus specifically downregulate expression from the major immediate-early promoter through a target located near the cap site. *J.Virol.* 1990; 64:6154-6165.
 88. Radsak K, Brucher KH, Britt W, Shiou H, Schneider D, Kollert A. Nuclear compartmentation of glycoprotein B of human cytomegalovirus. *Virology* 1990; 177:515-22.
 89. Stinski MF, Mocarski ES, Thomsen DR, Urbanowski ML. Membrane glycoproteins and antigens induced by human cytomegalovirus. *J.Gen.Virol.* 1979; 43:119-29.
 90. Kari B, Radeke R, Gehrz R. Processing of human cytomegalovirus envelope glycoproteins in and egress of cytomegalovirus from human astrocytoma cells. *J.Gen.Virol.* 1992; 73:253-60.
 91. Price GPA, Schrier RD, Oldstone MBA. Cytomegalovirus infects human lymphocytes and monocytes: virus expression is restricted to immediate-early gene products. *Proc.Natl.Acad.Sci.USA* 1984; 81:6134-6138.
 92. DeMarchi JM. Nature of the block in the expression of some early virus genes in cells abortively infected with human cytomegalovirus. *Virology* 1983; 129:287-97.
 93. Jeang KT, Chin G, Hayward GS. Characterization of cytomegalovirus immediate early genes. I. Nonpermissive rodent cells overproduct hIE94K protein from CMV (Colburn). *Virology* 1982; 121:393-403.
 94. Lafemina RL, Hayward GS. Differences in cell-type-specific blocks to immediate early gene expression and DNA replication of human, simian and murine cytomegalovirus. *J.Gen.Virol.* 1988; 69:355-74.
 95. Wu T-C, Lee WA, Pizzorno MC, Au W-C, Chan Y-J, Hruban RH, Hutchins GM, Hayward GS. Localization of the human cytomegalovirus 2.7-kb major early β -gene transcripts by RNA in situ hybridization in permissive and nonpermissive infections. *A.J.P.* 1992; 141:1247-1254.
 96. Ibanez CE, Schrier R, Ghazal P, Wiley C, Nelson JA. Human cytomegalovirus productively infects primary differentiated macrophages. *J.Virol.* 1991; 65:6581-6588.
 97. Lathey JL, Spector SA. Unrestricted replication of human cytomegalovirus in hydrocortisone-treated macrophages. *J.Virol.* 1991; 65:6371-6375.
 98. Tanaka J, Ogura T, Sato H, Hatano M. Establishment and biological characterization of an in vitro human cytomegalovirus latency model. *Viol.* 1987; 161:62-72.
 99. Cockley KD, Rapp F. Analysis of long-term human cytomegalovirus latency in vitro. *Intervirology* 1986; 26:129-139.
 100. Kondo K, Mocarsky ES. Cytomegalovirus latency and latency-specific transcription in hematopoietic progenitors. *Scand. J. Infect. Dis. Suppl.* 1995; 99:63-67.
 101. Kondo K, Xu J, Mocarski ES. Human cytomegalovirus latent gene expression in granulocyte-macrophage progenitors in culture and in seropositive individuals. *Proc. Natl. Acad. Sci. USA* 1996; 93:11137-11142.
 102. Sinclair J, Sissons P. Latent and persistent infections of monocytes and macrophages. *Interviol.* 1996; 39:193-301.
 103. Mendelson M, Monard S, Sissons P, Sinclair J. Detection of endogenous human cytomegalovirus in CD34+ bone marrow progenitors. *J.Gen.Virol.* 1996; 77:3099-102.
 104. Andersen CB. Detection of cytomegalovirus-infected cells in autopsy material by in situ hybridization. *A.P.M.I.S.* 1990; 98:363-368.
 105. Borisch B, Jahn G, Scholl BC, Filger-Brillinger J, Heymer B, Fleckenstein B,

- Muller-Hermelink HK. Detection of human cytomegalovirus DNA and viral antigens in tissues of different manifestations of CMV infection. *Virch.Arch.B.Cell.Pathol.* 1988; 55:93-99.
106. Wolber RA, Lloyd RV. Cytomegalovirus detection by nonisotopic in situ DNA hybridization and viral antigen immunostaining using a two-color technique. *Hum.Path.* 1988; 19:736-741.
107. Meyerson D, Hackman RC, Nelson JA, Ward DC, McDougall JK. Widespread presence of histologically occult cytomegalovirus. *Hum.Path.* 1984; 15:430-439.
108. Weiss LM, Movahed LA, Berry GJ, Billingham ME. In situ hybridization studies for viral nucleic acids in heart and lung allograft biopsies. *A.J.P.* 1990; 93:675-679.
109. Bij van der W, Schirm J, Torensma R, Son van W, Tegzess AM, Hauw The T. comparison between viraemia and antigenaemia for detection of cytomegalovirus in blood. *J.Clin.Microbiol.* 1988; 26:2531-2537.
110. Dankner WM, McCutchan JA, Richman DD, Hirata K, Spector SA. Localization of human cytomegalovirus in peripheral blood leucocytes by in situ hybridization. *J.Infect.Dis.* 1990; 161:31-36.
111. Laer von D, Serr A, Meyer-koning U, Kirste G, Hufert T, Haller O. Human cytomegalovirus immediate early and late transcripts are expressed in all major leukocyte populations in vivo. *J.Infect.Dis.* 1995; 172:365-370.
112. Boldogh I, Beth E, Huang ES, Kyalwazi SK, Giraldo G. Kaposi's sarcoma: detection of CMV DNA, CMV RNA, and CMNA in tumor biopsies. *Int.J.Cancer* 1981; 28:469-474.
113. Moore PS, Chang Y. Detection of herpesvirus-like DNA sequences in Kaposi's sarcoma in patients with and those without HIV infection. *N.Engl.J.Med.* 1995; 332:1181-1185.
114. Cesarman E, Chang Y, Moore PS, Said JW, Knowles DM. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. *N.Engl.J.Med.* 1995; 332:1186-1191.
115. David D, Ravid Z, Morag A. Detection of human cytomegalovirus DNA in human tonsillar lymphocytes. *J.Med.Virol.* 1987; 23:383-391.
116. Mathijs J-M, Rawlinson WD, Jacobs S, Bilous AM, Milliken JS, Dowton DN, Cunningham AL. Cellular localization of human cytomegalovirus reactivation in the cervix. *J.Infect.Dis.* 1991; 163:921-922.
117. Yu A-M, Song R-L, Yu Z, Chen Y-P, Wang F-Z, Na L-X, Xie G-L, Yang M-J. detection of human cytomegalovirus antigen and DNA in lymph nodes and peripheral blood mononuclear cells of patients with angioimmunoblastic lymphadenopathy with dysproteinemia. *Arch.Pathol.Lab.Med.* 1992; 116:490-494.
118. Fabricant CG, Fabricant J, Milnick CR, Litrenta MM. Herpes virus-induced atherosclerosis in chickens. *Fed.Proc.* 1983; 42:2476-2479.
119. Muscari A, Bozzoli C, Gerratana C, Zaca F, Rovinetti C, Zauli D, La Placa M, Puddu P. Association of serum IgA and C4 with severe atherosclerosis. *Atheroscler.* 1988; 74:179-186.
120. Grattan MT, Moreno-Cabral CE, Starnes VA, Oyer PE, Stinson EB, Shumway NE. Cytomegalovirus infection is associated with cardiac allograft rejection and atherosclerosis. *J.A.M.A.* 1989; 261:3561-3566.
121. Melnick JL, Petrie BL, Dreesman GR, Burek J, McCollum CH, Debakey ME. Cytomegalovirus antigen within human arterial smooth muscle cells. *Lancet* 1983; ii:644-647.
122. Melnick JL, Hu C, Burek J, Adam E, Debakey ME. Cytomegalovirus DNA in arterial walls of patients with atherosclerosis. *J.Med.Virol.* 1994; 42:170-174.
123. Speir E, Modali R, Huang ES, Leon MB, Shawl F, Finkel T, Epstein E. Potential role of human cytomegalovirus and p53 interaction in coronary restenosis. *Science* 1994; 265:391-394.

124. Kraat YJ, Hendrix MGR, Wijnen RMH, Peltenburg HG, Hooff van JP, Geelen JLMC, Bruggeman CA. Detection of latent human cytomegalovirus in organ tissue and the correlation with serological status. *Transplant.Int.* 1992; 5 (suppl.1):S615-616.
125. Minton EJ, Tysoe C, Sinclair JH, Sissons JGP. Human cytomegalovirus infection of the monocyte/macrophage lineage in bone marrow. *J.Virol.* 1994; 68:4017-4021.
126. Taylor-Wiedeman J, Hayhurst GP, Sissons JGP, Sinclair JH. Polymorphonuclear cells are not sites of persistence of human cytomegalovirus in healthy individuals. *J.Gen.Virol.* 1993; 74:265-268.
127. Zhang LJ, Hanff P, Rutherford C, Churchill WH, Crumpacker CS. Detection of human cytomegalovirus DNA, RNA, and antibody in normal donor blood. *J.Infect.Dis.* 1995; 171:1002-6.
128. Bitch A, Kirchner H, Dupke R, Bein G. Failure to detect human cytomegalovirus DNA in peripheral blood leucocytes of healthy blood donors by the polymerase chain reaction. *Transfusion* 1992; 32:612-617.
129. Urushibara N, Kwon KW, Takahashi TA, Sekiguchi S. Human cytomegalovirus DNA is not detectable with nested double polymerase chain reaction in healthy blood donors *Vox Sang* 1995; 68:9-14.
130. Staczek J. Animal cytomegaloviruses. *Microbiol.Rev.* 1990; 54:247-265.
131. Bruggeman CA, Li F, Stals FS. Pathogenicity: animal models. *Scand.J.Infect.Dis.* 1995; Suppl 99:43-50.
132. Collins TM, Quirk MR, Jordan MC. Bephasic viremia and viral gene expression in leukocytes during acute cytomegalovirus infection of mice. *J.Virol.* 1994; 68:6305-11.
133. Stoddart CA, Gardin RD, Boname JM, Manning WC, Abenes B, Mocarski ES. Peripheral blood mononuclear phagocytes mediate dissemination of murine cytomegalovirus. *J.Virol.* 1994; 68:6243-53.
134. Bale JF Jr, O'Neil ME. Detection of murine cytomegalovirus DNA in circulating leucocytes harvested during acute infection of mice. *J.Virol.* 1989; 63:2667-73.
135. Shanley JD, Biczak L, Forman S. Acute murine cytomegalovirus infection induces lethal hepatitis. *J.Infect.Diseas.* 1993; 167:264-9.
136. Mercer JA, Wiley CA, Spector DH. Pathogenesis of murine cytomegalovirus infection: identification of infected cells in the spleen during acute and latent infections. *J.Virol.* 1988; 62:987-97.
137. Abecasis MM, Jiang X, O'Neil ME, Bale JF Jr. Detection of murine cytomegalovirus (MCMV) DNA in skin using the polymerase chain reaction (PCR). *Microb.Pathogenesis* 1993; 15:17-22.
138. Keil GM, Fibi MR, Koszinowski UH. Characterization of the major immediate early polypeptides encoded by murine cytomegalovirus. *J.Virol.* 1985; 54:422-428.
139. Jordan MC, Pomeroy C. Latent cmv infection in the mouse. *Transplan.Proc.* 199 ; 23 (Suppl3).
140. Klotman MA, Henry SC, Greene RC, Brazy OC, Klotman PE, Hamilton JD. Detection of mouse cytomegalovirus nucleic acid in latently infected mice by in vitro enzymatic amplification. *J.Infect.Dis.* 1990; 161:220-225.
141. Balthesen M, Messerle M, Reddehase MJ. Lungs are a major organ site of cytomegalovirus latency and recurrence. *J.Virol.* 1993; 67:5360-5366.
142. Yuhasz SA, Dissette VB, Cook ML, Stevens JG. Murine cytomegalovirus is present in both chronic active and latent states in persistently infected mice. *Virology* 1994; 202:272-280.
143. Collins T, Pomeroy C, Jordan MC. Detection of latent cytomegalovirus DNA in divers organs of mice. *J.Infect.Dis.* 1993; 168:725-729.
144. Henry SC, Hamilton JD. Detection of murine cytomegalovirus immediate early 1

- transcripts in the spleens of latently infected mice. *J.Infect.Dis.* 1993; 167:950-954.
145. Bruggeman CA, Meyer H, Bosman F, van-Boven CPA. Biology of ratcytomegalovirus infection. *Intervirology* 1985; 24:1-9.
 146. Meijer H, Dreesen JCFM, van-Boven CPA. Molecular cloning and restriction endonuclease mapping of the rat cytomegalovirus genome. *J.Gen.Virol.* 1986; 67:1327-1342.
 147. Beuken E, Slobbe R, Bruggeman CA, Vink C. Cloning and sequence analysis of the genes encoding DNA polymerase, glycoprotein B, ICP 18.5 and major DNA-binding protein of rat cytomegalovirus. *J.Gen.Virol.* 1996; 77:1559-1562.
 148. Vink C, Beuken E, Bruggeman CA. Structure of the rat cytomegalovirus genome termini. *J.Virol.* 1996; 70:
 149. Bruggeman CA, Reactivation of latent CMV in the rat. *Transplant.Proc.* 1991; 23:22-24.
 150. Bruning JH, Bruggeman CA, van-Boven CPA, van-Breda-Vriesman PJ. Passive transfer of cytomegalovirus by cardiac and renal organ transplants in a rat model. *Transplantation* 1986; 41:695-698.
 151. Bruning JH, Bruggeman CA, van-Boven CPA, van-Breda-Vriesman PJ. Reactivation of latent rat cytomegalovirus by a combination of immunosuppression and administration of allogeneic immunocompetent cells. *Transplantation* 1989; 47:917-918.

CHAPTER 3

The presence of cytomegalovirus nucleic acids in arterial walls of atherosclerotic and non atherosclerotic patients

M.G.R. Hendrix
P.H.J. Dormans
P. Kitslaar
F. Bosman
C.A. Bruggeman

American Journal of Pathology 1989; 134:1151-1157.

Abstract

The presence of cytomegalovirus (CMV) nucleic acids was demonstrated in abdominal aorta's and femoral arteries of patients with and without atherosclerosis by dot-blot and in situ DNA hybridization using a DNA probe derived from immediate early genomic regions. Viral antigens could not be detected by immunohistochemistry and infectious virus could not be recovered from the arterial wall by virus isolation techniques. The high percentage (55%) of vascular wall specimen containing CMV nucleic acids, in atherosclerotic as well as in control material and the location of CMV containing cells in arteries without gross changes indicative of atherosclerosis suggest that the human arterial wall may be a site of latency for this virus.

Introduction

Infections with cytomegalovirus (CMV), a member of the herpesvirus group, are common. A characteristic feature of these viruses is that they persist in the host after primary infection and frequently reactivate from this latent state. This is clearly demonstrated in renal transplant recipients who almost invariably show evidence of active CMV infection (1).

Recently, several reports have suggested a role for CMV in the etiology of atherosclerosis. Studies in an animal model (chicken) have shown that infection with Marek's disease virus (MDV), a herpes virus, results in an extensive atherosclerosislike disease in normocholesterolemic chickens. Using in situ DNA hybridization techniques it was possible to show that MDV nucleic-acid sequences are present in the arterial walls of the infected chickens (2).

In humans the presence of herpes viruses in the arterial wall was also demonstrated by DNA in situ hybridization and immunohistochemical techniques. Benditt and coworkers (3) detected herpes simplex virus (HSV) by in situ DNA hybridization of aortic wall specimens obtained from patients undergoing coronary bypass surgery. Melnick et al. (4) described the presence of CMV antigens in smooth muscle cells cultured from tissues obtained from patients undergoing endarterectomy or coronary bypass surgery. Four years later these findings were confirmed by the same group using in situ DNA hybridization techniques (5). Recently the presence of HSV and CMV in the coronary arteries and thoracic aortas of young trauma victims without overt heart disease could be demonstrated by in situ DNA hybridization and immunohistochemical techniques (6). Most of these reports focus on the presence of CMV in arterial tissues obtained from patients with symptomatic atherosclerotic disease of the coronary and/or carotid arteries. It remains possible that the presence of CMV nucleic acids in atherosclerotic arterial walls reflects a site of latency as proposed by others (4,5,6) and that the virus will also be present in healthy arterial vessels.

In this report we describe the presence of CMV in arterial wall tissues in patients with overt atherosclerosis as well as trauma victims and patients without visible atherosclerosis at autopsy. In this study virus isolation, immunohistochemistry and DNA hybridization techniques with a probe derived from Immediate Early genomic regions were used.

Materials and methods

Patients and controls

To study the presence of CMV in the arterial wall, tissue samples were obtained from the femoral artery or abdominal aorta of CMV-seropositive patients undergoing recon-

structive vascular surgery for atherosclerotic disease. The patients received femoropopliteal bypass grafts or synthetic bifurcation grafts. Controls consisted of CMV-seropositive patients on whom autopsy was performed. Only patients dying of non-atherosclerotic disease and with maximally grade I atherosclerosis of the aorta as shown at autopsy were included. In addition, a small group of controls consisting of young trauma victims available for donor nephrectomy, were included in this study. In the control groups, tissue samples were obtained from the abdominal aorta.

Virus isolation

A 10% (wt/vol) homogenate of arterial wall specimens was inoculated onto monolayers of diploid human embryonic fibroblasts (HEF, Flow 2002). Inoculation was performed under a centrifugal force of 1000g for 1 hour at 37°C. Subsequently, cultures were placed on maintenance medium consisting of Eagle's minimal essential medium (MEM, Gibco, Grand Islands, NY) containing 10% fetal calf serum (FCS, Gibco) and antibiotics. The cultures were incubated at 37°C and examined microscopically twice weekly during a period of 5 weeks for the development of a cytopathologic effect (CPE). After 1 and 5 weeks the presence of viral antigens in the monolayer was analyzed using the indirect immunofluorescence technique. For the detection of viral antigens monoclonal antibodies (Dupont, Wilmington, DE) directed against CMV immediate early antigens (IEA) were used.

Fixation of tissues

Arterial tissues for in situ hybridization were fixed in a modified Carnoy's solution containing 73.5% absolute ethanol, 24.5% glacial acetic acid, and 2% formaldehyde (37%). After overnight incubation at 4°C the tissues were rehydrated by successive 30-minute washes in graded alcohols (100, 95, 85, 80 and 70% ethanol, respectively). Final storage of specimens was in 70% ethanol at 4°C. Arterial specimens for dot-blot hybridization were finally minced, placed in RPMI-1640 (Gibco) medium and an equal volume of 95-100% ethanol was added slowly under gentle agitation. Fixed tissues were stored at 4°C in 50% ethanol.

Monoclonal antibodies

Monoclonal antibody H88 directed against immediate early antigens of CMV, prepared following standard procedures, was used in this study. The method employed for producing hybridomas secreting CMV antibodies have been described for rat CMV (RCMV) in detail previously (7). Briefly, BALB/C mice were immunized with sonicated suspension of CMV strain AD169 infected HEF. After 4 weeks their spleen cells were fused with mouse myeloma cells of the SP 2/0 Ag-14. Hybrid cells were selected in hypoxanthine-aminopterin-thymidine medium and CMV antibody producing clones were identified by enzymelinked immunosorbent assay and indirect immunofluorescence staining on CMV infected HEF monolayers as described previously for RCMV (7). Selection of the clones producing antibodies to the immediate early (IE) antigens of CMV were done using infected HEF blocked with cycloheximide and actinomycin D to only induce immediate early antigens (12).

Monoclonal antibody H88 which selectively precipitates a 68 KD protein from infected fibroblasts was used in this study. This monoclonal antibody gives a uniform nuclear fluorescence in CMV infected fibroblasts within as little as 12h after infection. No cross reactivity with other herpes viruses could be detected. High titer mouse ascitic fluid was produced by infecting the antibodyproducing clone intraperitoneally into pristane treated mice.

Immunohistochemistry

Thin tissue sections of approximately 4 μ m mounted on glassslides were deparaffinized

and washed in 90% ethanol, followed by blocking of endogenous peroxidase activity by incubation in 100% methanol with 0.3% H₂O₂ for 30 min.

The sections were then washed in phosphate-buffered saline (PBS), preincubated with PBS containing 2% Bovine Serum Albumin (BSA) for 30 minutes, followed by incubation for 60 minutes with one of the mouse McAb's, washed again in PBS for 5 minutes, followed by incubation with biotinylated affinity purified sheep anti-mouse Ig (Amersham) 1:200 in PBS containing 2% BSA for 60 minutes, and washed again for 5 minutes in PBS followed by incubation with Biotin Streptavidin HRP-complex (Amersham) 1:400 in PBS containing 2% BSA for 45 minutes.

After washing again in PBS the sections were incubated for 10 minutes in diaminobenzidine-4HCl-substrate 0.05% in Tris-HCl buffer pH7.4, containing 0.002% H₂O₂. The color reaction was stopped by washing in PBS, followed by counterstaining for 1 minute in hematoxylin, dehydration and mounting in Entellan (Merck, Darmstadt, FRG).

Fig. 1 Restriction endonuclease cleavage map of the cloned EcoR1-J fragment, and the subcloned 7.0 kb pES fragment. The topline represents the 236 kb genome of HCMV strain AD169 and shows the location of the EcoR1-J fragment in the prototype orientation. pES indicates the cloned EcoR1-J-Sal1 fragment. P(PvuII); E(EcoR1); B(BamH1); Bg(BglII); Sa(Sal1); Sp(Sph1) represent the restriction sites present in the pES fragment.

DNA probes

Plasmid pBR 328 containing the 7.0 Kb ES fragment, an EcoR1-Sal1 subdigestion of the EcoRI-J Fragment of CMV strain AD 169 (Fig. 1) was provided by Dr. J. Geelen, University of Amsterdam, The Netherlands. Plasmid was transfected into an Escherichia coli mutant J.M. 109 and pure ES fragment DNA was recovered using standard procedures (8). Controls consisted of total CMV strain AD 169 DNA, total rat cytomegalovirus (RCMV) DNA (9), and plasmid pBR 328 DNA without inserts. In all DNA biotin was inserted by incorporation of biotin-11-dUTP (Bethesda Research Laboratories, Bethesda, MD) using a random primed DNA labeling kit (Boehringer). The specificity of the biotinylated DNA probes was evaluated in uninfected HEF, HEF infected with CMV laboratory strain AD 169, and in arterial samples using both dot blotting and in situ hybridization procedures. The in situ hybridization procedures also were performed without DNA probes to evaluate non-specific reactions.

Dot blot hybridization

Extraction of cellular DNA was performed essentially as described previously (10). After extensive washings the ethanol fixed tissues were digested overnight at 50°C in a

mixture containing 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 100 mM NaCl, 0.2 mg/ml proteinase K (Boehringer), and 10 mg/ml SDS. Proteins were removed using successive phenol-chloroform and chloroform extractions and DNA was recovered by ethanol precipitation. RNA was digested by incubating the precipitate during 30 min at 37°C in a mixture of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA and 75 µg RNase A (Sigma Chemical Co., St. Louis, MO). DNA was again ethanol precipitated, air dried, and redissolved in distilled water. DNA was quantified by spectrophotometry at 260 and 280 nm. For hybridization 5-10 µg DNA (NaOH denatured) was spotted onto nitrocellulose paper (BA 85, Schleicher and Schull). After incubation at 80°C for 2 hours filters were prehybridized for 4 hours at 42°C in a mixture containing 50% deionised formamide, 5x SSC, 5x Denhardt's solution, 25 mM sodium phosphate buffer, pH 6.5, and 0.5 mg/ml heat denatured herring sperm DNA. After this period the prehybridization solution was replaced with 150 ng/ml heat denatured dUTP probe and 5% (wt/vol) dextran sulphate in the same solution. Hybridization was carried out at 42°C for 12 hours in a roller drum at 1 to 2 rpm. Filters were washed in two changes of 2x SSC, 0.1% SDS at room temperature for 5 minutes each, twice in 0.2x SSC, 0.1% SDS at room temperature for 5 minutes each, twice in 0.16x SSC, 0.1% SDS at 55°C for 15 minutes each and finally once in 2x SSC at room temperature for 1 minute. Visualisation of successful hybridization was performed with the BLU gene kit (Bethesda Research Laboratories). The specificity of the biotinylated probes was evaluated with DNA extracted from CMV strain AD169 infected HEF and uninfected HEF spotted onto nitrocellulose paper. Only ES probe and total CMV probe showed hybridization with DNA extracted from infected HEF but not with DNA extracted from uninfected HEF. RCMV and plasmid probes did not show hybridization.

In situ hybridization

Arterial specimens fixed in a modified Carnoy's solution were embedded in paraffin blocks using routine histopathological procedures. Sections 3 to 4 µm thick were cut from these blocks and placed on chromium-alum-gelatin coated and glutaraldehyde activated slides (11). After overnight incubation at 52-54° sections were dewaxed in xylene, washed once in 100% ethanol and air dried.

Aldehyde groups were inactivated by incubating the slides with 1% ammonium chloride in PBS. Proteolytic digestion with proteinase K (Boehringer) (2.5-20 µg/ml) in 20 mM Tris-HCl, pH 7.4 containing 2 mM CaCl₂ was carried out for 30 minutes at 37°C and the reaction was subsequently stopped by washing the slides with PBS containing 2 mg/ml glycine. Slides were then dehydrated by successive washings in graded alcohols (30, 60, 80, 95 and 100% ethanol, respectively) and air dried. When needed, RNA digestion was carried out by incubating the slides with 100 µg/ml RNase A ((Sigma) in PBS at 37°C, 1 hour and the reaction was stopped by 2 successive washings in PBS. Slides were again dehydrated by successive washings in graded alcohols. Immediately after these pretreatment steps, samples were overlaid with 48% deionised formamide, 10% dextran sulphate, 5x SSC, 5x Denhardt's solution, 50 mM sodium phosphate, pH 6.4, 0.1 µg/ml herring sperm DNA, and 200 µg/ml probe DNA covered with a silicon-coated coverslip and sealed with rubber cement. Cellular and probe DNA were denatured by heating the slides to 83°C and immediately immersing them in ice-cold water. Hybridization was carried out overnight at 42°C. Washing procedures and visualisation of formed hybrids was performed as described for dot blot hybridization.

The specificity of the biotinylated probes was evaluated on CMV strain AD169 infected HEF monolayers and on uninfected HEF monolayers. Only ES probe and total CMV probe showed hybridization with infected HEF monolayers, but not with uninfected HEF monolayers. RCMV and plasmid probes never showed hybridization.

Statistical analysis

Statistical analysis was carried out using Fisher's exact tests. Results were considered significant when $p < 0,01$.

Results

Patients and controls

Tissue samples were obtained from 44 patients undergoing vascular surgery (Table 1). Twenty-two samples were taken from the abdominal aorta (20 men, 2 women) and 22 samples were taken from the femoral artery (10 men, 12 women). The mean age of these patients was 66 years. Controls consisting of eight trauma victims (6 men, 2 women) with a mean age of 32 years were used. Arterial samples were taken from the abdominal aorta during donor nephrectomy.

The autopsy-control series contained 23 patients (10 men, 13 women) with a mean age of 69 years. The abdominal aorta samples were taken within 12 hours after death. Mean ages of the atherosclerotic and autopsy control group were in the same range, as was the distribution among the sexes. Statistical analysis of these data showed no significant differences, so the groups were suitable for comparison. The mean age of the trauma victim group, however, was too low for comparison.

Table 1. Characteristics of patients series under investigation

Patients	Biopsies	Mean age (years $\pm$ SD)	Range (years)	Men	Women
Grade III atherosclerosis (surgical patients)	Femoral artery	70 $\pm$ 9.5	55-88	10(45%)	12(55%)
	Abdominal aorta	62 $\pm$ 10.0	44-80	20(90%)	2(10%)
	Abdominal aorta	69 $\pm$ 13.6	45-91	10(43%)	13(57%)
No atherosclerosis (autopsy)	Abdominal aorta	32 $\pm$ 18.0	9-60	6(75%)	2(25%)
Trauma victims					

Virus isolation

Virus isolation experiments were performed on fresh arterial samples only. In none of the tested samples was a cytopathological effect (CPE) observed in the HEF monolayers during a 5-week period. Using the indirect immunofluorescence technique with monoclonal antibodies directed against CMV, immediate early antigens (IEA) viral proteins were not detected in the HEF monolayers, indicating the absence of infectious virus.

Immunohistochemistry

Immunohistochemical studies were performed on all arterial samples. Immunoreactivity was not observed in any of the tested samples using McAbs against IEA.

Dot blot hybridization

Using the dot blot hybridization technique (Table 2) CMV nucleic acid sequences were detected in 4 of 22 (18%) samples of the femoral artery and in 7 of 22 (32%) samples of the abdominal aorta of patients with severe atherosclerosis. Statistical analysis revealed this difference between the two groups to be not significant ($p < 0,447$). By evaluating the extracted DNA in the samples obtained from the abdominal aorta of patients without signs of atherosclerosis, CMV nucleic acid sequences were detected in 5 of 23 (22%) cases, which is not statistically different from the atherosclerosis samples ($p < 0,479$). Of samples from trauma victims, 4 of 8 (50%) were positive for CMV DNA

but this group was too small for statistical analysis. Thus, we could conclude that 30% of the samples tested contained CMV nucleic acids using this technique.

Table 2. Results of dot blot and in situ DNA hybridization of arterial tissues

Patients	Biopsies	CMV hybridization dot blot number positive/ total number (%)	CMV hybridization in situ number positive/ total number (%)
Grade III atherosclerosis (surgical patients)	Femoral artery	4/22 (18%)	9/22 (41%)
	Abdominal aorta	7/22 (32%)	10/22 (45%)
No atherosclerosis (autopsy)	Abdominal aorta	5/23 (22%)	17/22 (77%)
	Abdominal aorta	4/8 (50%)	5/8 (63%)

Figure II.

Photomicrographs of in situ hybridization of sections of arterial walls using CMV-specific probes and haematoxylin-eosin (H&E) staining of sequential sections of arterial walls of two patients (A and B). A1: An in situ hybridization of the intima and media of an arterial wall. The dark spots (arrow) represent the hybridization (100x). A2: Magnification of A1 showing discrete nuclear hybridization (250x). A3: sequential section of A2, stained with H&E, showing a normal arterial architecture. B1: Another example of an in situ hybridization. B2: Sequential section stained with H&E (520x)

In situ hybridization

In situ DNA hybridization (Fig. 2) of sections of arteries obtained from patients with severe atherosclerosis showed that 9 of 22 (41%) biopsies of femoral arteries and 10 of 22 (45%) biopsies of abdominal aortas contained CMV nucleic acid sequences; statistical analysis showed no significant differences between these groups ($p < 0,220$). Evaluation of the samples of the abdominal aorta of patients without atherosclerosis at autopsy revealed that 17 of 22 (77%) contained CMV DNA, which is not statistically different ($p < 0,240$) from the group with atherosclerosis. Five of 8 (63%) samples obtained from the abdominal aorta of trauma patients were positive for CMV nucleic acids. However, the number of trauma victims was too small for statistical analysis. Thus, we could conclude that 55% of the samples tested contained CMV nucleic acids using this technique.

Tissue samples that showed extensive in situ hybridization reactivity were analyzed further by RNA digestion before in situ DNA hybridization. After RNase treatment the tissue sections showed a less intense hybridization reaction, and in some cases the reactivity disappeared altogether.

Histologically, the CMV nucleic acid sequences detected with the ES probe were located mainly in apparently normal arterial tissue without an inflammatory reaction or atheromatous changes. Sequential sections, stained with haematoxylin and eosin, showed that the DNA hybridization reactivity was located mainly in smooth muscle cells in the arterial media.

Discussion

This study has demonstrated the presence of CMV nucleic acid sequences in biopsies of femoral arteries and abdominal aortas of atherosclerosis patients as well as controls. CMV nucleic acids were distributed equally among patients with and without this disease. No differences were observed in CMV distribution between biopsies taken from femoral arteries or from abdominal aortas.

The percentage of samples containing CMV nucleic acid (30%) by dot blot hybridization techniques equals the percentage found by others using in situ DNA hybridization techniques (5, 6) or by immunohistochemistry (4). Using in situ hybridization, however, we found that a considerably higher percentage (55%) of the samples investigated contained CMV nucleic acids. There are two possible explanations for this phenomenon: First, our in situ DNA hybridization technique did not include RNase treatment of the tissue sections. It is therefore likely that the probe DNA formed not only DNA-DNA hybrids but also RNA-DNA hybrids (11). The finding that tissue samples that showed extensive in situ hybridization reactivity showed a less intense hybridization reaction and in some cases became negative after RNase treatment seems to corroborate this possibility. It is therefore likely that in situ mRNA-DNA hybrids contribute significantly to the obtained reactivity. In contrast, extraction of DNA for dot blot hybridization always included RNase treatment and consequently only the samples sufficiently rich in viral DNA showed hybridization reactivity using this technique.

Second, in our view the most likely explanation of the observed difference is the choice of the CMV DNA probe, which was prepared from the EcoR1-J fragment of CMV strain AD169, containing sequences coding for immediate early transcripts (12) (Fig.1). Recently, latency associated transcripts (LAT) were described in neurons during latent Herpes Simplex Virus (HSV) infection (14, 15), originating from genomic regions coding for immediate early transcripts of HSV (16, 17, 18). These HSV mRNA transcripts could be detected by DNA probes prepared from the immediate early genomic regions (14). In CMV the immediate early transcripts might therefore not only be transcribed during reproductive viral cycles (13), but also during latent infections. The high percentage

(55%) of arterial samples containing CMV nucleic acids in this study can therefore be explained by the use of the ES probe, because in other studies (5, 6) probes coding for nonimmediate early regions were used. Together with the negative results from the virus isolation and the negative results from the immunohistochemical studies, we conclude that the arterial wall may be a site of latency for CMV, as already proposed by others (4, 5, 6).

Although the present results do not suggest a direct role for CMV in the pathogenesis of atherosclerosis, the presence of (latent) virus in the vessel wall might contribute to the process of atherogenesis. The ability to reactivate from the latent status leading to a local lytic infection in the vessel wall might result in repeated local inflammatory reactions eventually leading to vascular injury. This injury, in cooperation with other established risk factors, could play a still unknown role in atherogenesis.

Further studies are needed to elucidate the factors leading to reactivation of the CMV and the development of atherosclerosis as a consequence of a local productive infection.

References

1. Ho m. Cytomegalovirus biology and infection. New York, Plenum Press, 1982
2. Fabricant CG, Fabricant J, Milnick CR, Litrenta MM. Herpesvirus-induced atherosclerosis in chickens. *Fed.Proc.* 1983;42:2476-2479
3. Benditt EA, Barret T, McDougall JK. Viruses in the etiology of atherosclerosis. *Proc Natl Acad Sci USA* 1983; 6386-6389.
4. Melnick JL, Petrie BL, Dreesman GR, Burek J, McCollum CH, Debakey ME. Cytomegalovirus antigen within human arterial smooth muscle cells. *Lancet* 1983, 2: 644-647.
5. Petrie BL, Melnick JL, Adam E, Burek J, McCollum CH, Debakey ME. Nucleic acid sequence of cytomegalovirus in cells cultured from human arterial tissue. *J Infect Dis* 1987, 155: 158-159.
6. Yamashiroga HM, Ghosh L, Yang R, Robertson L. Herpes viridae in the coronary arteries and aorta of young trauma victims. *Am J Path* 1988, 130: 71-79.
7. Bruning JH, Debie WHM, Dormans PHJ, Meijer H, Bruggeman CA. The development and characterization of monoclonal antibodies against rat cytomegalovirus induced antigens. *Arch.Virol.* 1987;94:55-70.
8. Birnboim HC, Doly J. A rapid alkaline extraction method for the isolation of plasmid DNA. *Nucl Acid Res* 1979, 7: 1513-1518.
9. Meyer H, Dreesen JCFM, van Boven CPA. Molecular cloning and restriction endonuclease mapping of rat cytomegalovirus genome. *J Gen Virol* 1986, 67: 1327-1342.
10. Smith LJ, Braylan RC, Natkis JE, Edmunson KB, Downing JR, Wakeland EK. Extraction of cellular DNA from human cells and tissues fixed in ethanol. *Anal.Biochem.* 1987;160:135-138.
11. Deatly AM, Spirack JG, Lavi E, Fraser NW. RNA from an immediate early region of the type I herpes simplex virus genome is present in the trigeminal ganglia of latently infected mice. *Proc.Natl.Acad.Sci.USA* 1987;84:3204-3208.
12. Boom R. Repression and activation of the human cytomegalovirus major immediate early transcription unit. PhD Thesis. Rodopi Press, Amsterdam, 1987.
13. Stinski MF, Thomsen DR, Stenberg RM, Goldstein LC. Organization and expression of the immediate early genes of the human cytomegalovirus. *J Virol* 1983, 46: 1-14.
14. Stevens JG, Haar L, Porter DD, Cook ML, Wagner EK. Prominence of the Herpes Simplex virus latency associated transcript in trigeminal ganglia from seropositive

- humans. *J Infect Dis* 1988, 158: 117-123.
15. Croen KD, Ostrove JM, Dragovic LJ, Smialek JE, Straus SE. Latent Herpes Simplex virus in human trigeminal ganglia. *N Engl J Med* 1987, 23: 1427-1432.
 16. Wagner EK, Devi-Rao G, Feldman LT, Dobson AT, Zhang Y, Flanagan WM, Stevens JG. Physical characterization of the Herpes Simplex virus. Latency-associated transcript in neurons. *J Virol* 1988, 62: 1194-1202.
 17. Deatly AM, Spivack JG, Lavi E, O'Boyle II DR, Fraser NW. Latent herpes simplex virus type 2 transcripts in peripheral and central nervous tissues of mice map to similar regions of the viral genome. *J.Virol.* 1988;62:749-756.
 18. Gordon YL, Johnson B, Romanowski E, Araulo-Cruz T. RNA complementary to herpes simplex type I ICPO gene demonstrated in neurons of human trigeminal ganglia. *J.Virol.* 1988;62:1832-1835.

CHAPTER 4

High prevalence of latently present cytomegalovirus in arterial walls of patients suffering from grade III atherosclerosis

M.G.R. Hendrix
M.M.M. Salimans
C.P.A. van Boven
C.A. Bruggeman

American Journal of Pathology 1990; 136:23-28.

Abstract

The presence of Cytomegalovirus (CMV) nucleic acids was demonstrated in arterial walls of patients with grade III and with maximally grade I atherosclerosis by dot blot and in situ DNA hybridization and by polymerase chain reaction (PCR) using probes and primers derived from Immediate Early (IE) and Late (L) genomic regions. The presence of the complete viral genome could be demonstrated by both dot blot DNA hybridization and PCR. IE mRNA but not L mRNA could be demonstrated by in situ DNA hybridization, indicating the presence of latent CMV in the human arterial wall. By PCR 90% of the samples obtained from atherosclerotic patients were shown to contain viral nucleic acids as compared to 53% of patients with maximally grade I atherosclerosis, thus substantiating a role for this virus in the pathogenesis of atherosclerosis.

Introduction

Recently several investigators were able to demonstrate the presence of Cytomegalovirus (CMV) nucleic acids and/or antigens in the human arterial wall by using DNA hybridization techniques (1) or immunohistochemistry (2). Most arterial specimen were taken from patients undergoing reconstructive vascular surgery for severe atherosclerotic disease, and so a role for the CMV in the pathogenesis of this disease was indicated (3). On the basis of these observations it was suggested that the human arterial wall might be a site of latency for the CMV (1, 2, 4). Local reactivation of this virus could then lead to vascular injury and consequently contribute to the development of atherosclerotic lesions. However, the studies published did not include an age and sex matched control group of patients with minimal or no signs of atherosclerosis.

In our laboratory we demonstrated the presence of CMV nucleic acids in the human arterial wall (5) by Dot blot and in situ DNA hybridization techniques. We didn't, however, observe any differences in the distribution of CMV nucleic acids in samples of patients with severe atherosclerosis or patients with maximally grade I atherosclerosis. A possible explanation for this observation is the relative insensitivity of the methods used. Only samples that were sufficiently rich in viral nucleic acids could be detected. More sensitive methods might still show a difference in the distribution of the virus among the patient groups investigated.

Using in situ DNA hybridization with a probe derived from Immediate Early (IE) genomic regions demonstrated that 50% of the samples tested contained CMV nucleic acids, which is considerably higher (5) than the percentages found by other researchers using various DNA probes (1, 3). We showed that mRNA-DNA hybrids contributed significantly to the hybridization reactivity observed. This finding that 55% of the arterial samples tested with the ES probe contained CMV nucleic acids shows that mRNA transcribed from IE genomic regions are not only present during reproductive viral cycles (6) but also during latent infections.

In this report we provide further evidence that the CMV is latently present in the human arterial wall by in situ DNA hybridization with a probe derived from genomic regions coding for structural proteins. We show also that the complete viral genome is conserved in the arterial wall by dot blot DNA hybridization and Polymerase Chain Reaction (PCR). Using the PCR we found that the prevalence of CMV nucleic acids in patients suffering from severe atherosclerosis is significantly higher than in patients with maximally grade I atherosclerosis.

Materials and methods

Patients and controls

The subjects of this study were as described previously (5). In brief, arterial biopsies were taken from CMV-seropositive patients undergoing reconstructive vascular surgery. Biopsies were taken from the abdominal aorta or from the femoral artery. CMV-seropositive controls consisted of age and sex matched patients with maximally grade I atherosclerosis as shown at autopsy. In this group biopsies were taken from the abdominal aorta. Autopsy was always performed within 12 hours after death.

In addition to our previous study, we increased the number of patients entering the control group.

Processing of tissue specimens

Arterial tissues for in situ and dot blot hybridization and for polymerase-chain reaction were processed as reported previously (5). For in situ DNA hybridization, tissue specimen were incubated overnight in a modified carnoy's solution, dehydrated by successive washes in graded alcohols, and embedded in paraffin, using routine histopathological procedures. For dot blot hybridization and PCR, DNA was extracted from the arterial specimen by proteolytic digestion and phenol-chloroform extraction followed by ethanol precipitation. DNA was quantified by spectrophotometry at 260 and 280 nM.

Hybridization probes

All enzymes were purchases from Boehringer-Mannheim (FRG). Enzyme reactions were performed under conditions recommended by the manufacturer. Recombinant plasmids were prepared by following published procedures (7) using vector pBR328.

Plasmid pES (Fig. 1) containing the 7,0 Kb ES fragment, an EcoRI-SalI subdigestion of the EcoRI-J fragment of CMV strain Ad 169, coding for the major Immediate Early (IE) protein (8) was kindly provided by Dr. J. Geelen (University of Amsterdam, the Netherlands).

Plasmid pBH (Fig. 1) containing the 2,9 Kb BH fragment, coding for the late nucleocapsid protein GP 64 (9, 10), was constructed from a HindIII-Bam HI subdigestion of cosmid PCM 1075. This cosmid was kindly provided by Prof.Dr. A.J. van der Eb (University of Leiden, the Netherlands). Both plasmids were transfected into *Escherichia coli* mutant JM 109 and by standard procedures (7, 11) pure ES and BH DNA was recovered. Controls consisted of total CMV strain AD169 DNA, total RCMV DNA (12) and plasmid pBR328 DNA without inserts. In all DNA biotin was inserted by incorporation of biotin-11-dUTP (Bethesda Research Laboratories) using a random primed DNA labeling kit (Boehringer-Mannheim).

Dot-blot hybridization

Spotting of cellular DNA on nitrocellulose paper, hybridization conditions, and visualization of successful hybridization were carried out as described previously (5).

The specificity of the biotinylated probes was evaluated with DNA extracted from CMV strain AD169 infected human embryonic fibroblasts (HEF) and uninfected HEF spotted onto nitrocellulose paper. Only ES probe, BH probe and total CMV probe showed hybridization with DNA extracted from infected HEF but not with DNA extracted from uninfected HEF. RCMV and plasmid probes never demonstrated hybridization.

Fig. 1 Restriction endonuclease cleavage map of CMV strain AD 169. The topline represents the HindIII digestion map in prototype arrangement (8) showing the positions of the HindIII-E fragment containing IE sequences and the Hind III-L fragment containing Late sequences. The left panel shows the position of the cloned ES fragment (7.0 KB) coding for the major IE protein (8) within the HindIII-E fragment relative to the EcoRI-J, c and d fragments. The right panel shows the position of the cloned BH fragment (2.9 KB) coding for the late nucleocapsid protein GP 64 (9-8) within the HindIII-L fragment relative to the BamHI-X, b and d fragments. H(HindIII); E(EcoRI); S(Sall); B(BamHI) represent restriction endonuclease cleavage sites.

In-situ hybridization

The in situ DNA hybridization protocol performed on tissue sections of arterial specimen has been described previously (5). The specificity of the biotinylated probes was evaluated on CMV strain AD169 infected HEF monolayers and on uninfected HEF monolayers. Only ES probe, BH probe and total CMV probe showed hybridization with infected HEF monolayers, but not with uninfected HEF monolayers. RCMV and plasmid probes never showed hybridization.

Oligomer synthesis

The oligomers used as primers in the polymerase chain reaction (PCR) were synthesized by Dr. H. Meijer (Department of Genetics and Cell Biology, University of Limburg, Maastricht, The Netherlands) on a DNA synthesizer (cyclone DNA synthesizer, Biosearch Inc., New Brunswick) by the fosfamidid method. The sequences for the oligomer set complementary to the major IE-gene were based on the sequence described by Akrigg et al. (13) and located in the insert of plasmid pES. The sequences are: 5-GGA-GAT-GTG-GAT-GGC-TT-G-TA-3 (IE₁ upstream primer) and 5-GAA-GGC-TGA-GTT-CTT-GGT-AA-3 (IE₂ downstream primer). The sequences for the oligomer set complementary to the nucleocapsid GP 64 gene correspond to published sequences (14) and are located in the insert of plasmid pBH. The sequences are 5-CCG-CAA-CCT-GGT-GCC-CAT-GG-3 (LA₁ upstream primer) and 5-CGT-TTG-GGT-TGC-GC-A-GCG-GG-3 (LA₂ downstream primer). The sizes of the PCR amplification products of the IE and GP 64 targets are 170 and 140 base pairs, respectively.

Polymerase chain reaction

Amplification of extracted cellular DNA was performed in a total volume of 30 μ l. The reaction mixture contained: 10 mM Tris-HCl (pH 9,6), 10 mM $MgCl_2$, 0,2 mg/ml BSA Boserol (Organon), 50 mM NaCl, 1 mM dATP, 1 mM dCTP, 1 mM dTTP, 1 mM dGTP (all nucleosides purchased from Promega), 0,5 μ g of each primer, 1 U cloned thermo-stable DNA polymerase from *Thermus Aquaticus* (Ampli-Taq, Perkin-Elmer-Cetus) and 2,5-5,0 μ g of sample DNA. The reaction mixture was overlaid with 20 μ l paraffin oil to prevent evaporation. The amplification reaction was performed on a DNA thermal cycler (Cetus). Each primer set was evaluated with regard to annealing temperature and the number of cycles required to achieve the highest signal-to-noise ratio when the products were evaluated by direct staining of 2% agarose (Seakem) gels. The reaction conditions that follow, were found to be optimal. After initial heating of the samples for 5 min at 94°C, 37 cycles were carried out. Each cycle was built up as follows: heating for 30 s at 94°C to denature the target DNA, then cooling to 59°C for 45 s to allow the primers and DNA to reanneal, and finally heating to 72°C for 120 s for primer extension. Fifteen μ l Of the reaction mixture was subjected to electrophoresis on 2% agarose (Seakem) gels containing 0,5 μ g/ml EtBr. The DNA was visualized by UV fluorescence. The specificity and sensitivity of both primer sets has been tested extensively by others (13, 14). No cross reactivity was observed between the IE primer set and plasmid pBH nor between the LA primer set and plasmid pES.

Also no reactivity was observed when testing both primer sets on DNA obtained from HEF or from leucocytes from healthy individuals. DNA samples which didn't show reactivity with the CMV primers used were further analysed with a primer set reactive with the β -globine gene (15) to check on DNA preservation. All samples tested this way did show an 110 bp amplification product of the β -globine gene proving that DNA preservation was sufficient for PCR analysis.

Statistical analysis

Statistical analysis was carried out using Fisher's exact tests. Results were considered significant when $p < 0,01$.

Results

Patients and controls

Tissue samples were obtained from 44 patients undergoing reconstructive vascular surgery (30 men, 14 women) for obstructive atherosclerotic disease. Twenty-two samples were taken from the femoral artery (10 men, 12 women) and 22 samples were taken from the abdominal aorta (20 men, 2 women). The mean age of all patients was 66 years (range 44-88).

The autopsy-control series contained 34 patients (16 men, 18 women) with a mean age of 69 years (range 45-91). In this group samples were taken from the abdominal aorta within 12 hours after death.

Mean ages of atherosclerotic and autopsy-control group were in the same range as was the distribution among the sexes.

Dot blot DNA hybridization

Using the dot-blot hybridization technique (Table 1) CMV nucleic acid sequences were detected in 11 of 44 (25%) samples obtained from patients with severe atherosclerosis using the ES probe, and in 4 of 14 (29%) samples of the same patient group using the BH probe. Statistical analysis revealed this difference between the two probes was not significant ($p = 0.5214$).

By evaluating the extracted DNA of the samples obtained from patients with maximally

grade I atherosclerosis, CMV nucleic acids were detected in 7 of 33 (21%) cases using the ES probe and in 4 of 23 (18%) cases using the BH probe which was not a statistically significant difference ($p = 0.5003$).

Also no statistical significant differences were observed in the distribution of CMV nucleic acids in samples of patients with or without atherosclerosis by using the ES probe ($p = 0.4565$) or by using the BH probe ($p = 0.343$).

Overall we could conclude that by using this technique 27% of the samples tested contained CMV nucleic acids regardless of the patient group or the probe used.

Table 1. Results of dot blot and in situ DNA hybridization with probes derived from immediate early (ES fragment) and late (BH fragment) genomic regions and of the polymerase chain reaction with primers reactive with immediate early (IE primers) and late (LA primers) genomic sequences.

Hybridization technique	Grade III atherosclerosis Nr. POS/ total nr. (%)	Max. grade I atherosclerosis Nr. POS/ total nr. (%)
Dot blot hybridization		
ES fragment	11/44 (25%)	7/33 (21%)
BH fragment	4/14 (29%)	2/23 (18%)
In situ hybridization		
ES fragment	18/41 (44%)	19/33 (58%)
BH fragment	5/41 (12%)	5/33 (15%)
Polymerase chain reaction		
IE primers	27/30 (90%)	18/34 (53%)
LA primers	27/30 (90%)	18/34 (53%)

In situ DNA hybridization

In situ DNA hybridization (Table 1) of sections of arteries obtained from patients with severe atherosclerosis showed that 18 of 41 (44%) contained CMV nucleic acids by using the ES probe. However, by using the BH probe only 5 of 41 (12%) samples obtained from the same patient group were shown to contain CMV nucleic acids. The differences found between these two probes are statistically significant ($p = 0.002$).

Evaluation of the samples obtained from patients with maximally grade I atherosclerosis revealed that 19 of 33 (58%) contained CMV nucleic acids by using the ES probe and only 5 of 33 (15%) by using the BH probe. The difference between these two probes was also statistically significant ($p = 0.0004$).

There was no difference in the distribution of CMV nucleic acids as demonstrated by in situ hybridization between patients with or without atherosclerosis by using the ES probe ($p = 0.175$) or by using the BH probe ($p = 0.485$).

Overall, we could conclude that 50% of the samples tested with the ES probe contained CMV nucleic acids, but by using the BH probe only 14% of these samples could be demonstrated to contain the Cytomegalovirus.

Polymerase chain reaction

The polymerase chain reaction performed on pure DNA extracted from arteries of patients suffering from severe atherosclerosis showed that 27 of 30 (90%) contained CMV nucleic acids regardless of the primer set used (Table 1).

The samples obtained from patients without atherosclerosis showed exactly the same reactivity with regard to the primer set used. Eighteen of 34 (50%) samples were demonstrated to contain CMV nucleic acids by both primer sets, one directed against the DNA coding for the major immediate early antigen and one directed against the DNA coding for the nucleocapsid protein GP 64.

Samples reactive with the IE primer set were also always reactive with the LA primer set. Negative samples did not react with the IE primer set or with the LA primer set.

We observed a great difference in the distribution of CMV nucleic acids obtained from

the two patient groups under investigation. Far more samples, 27 of 30 (90%), obtained from patients with severe atherosclerosis contained CMV nucleic acids as compared to the samples obtained from patients with maximally grade I atherosclerosis, 18 of 34 (53%) ($p = 0.001$). Overall we could conclude that by using this technique 90% of the atherosclerotic patients contain CMV nucleic acids in their arterial walls as compared to 53% of the patients with maximally grade I atherosclerosis, regardless of the primer set used.

Discussion

In this study we demonstrated that the percentage of arterial specimens obtained from patients with or without atherosclerosis containing CMV nucleic acids depended highly on the DNA detection method and probes used. With a crude method like dot blot DNA hybridization only low percentages of samples (27%) containing the virus could be detected, regardless of the probes used. However, by using *in situ* DNA hybridization techniques, a higher percentage of samples could be shown to contain the viral nucleic acids, depending on the probes used. Probes directed against IE genomic sequences gave higher yields (50%) than probes directed against structural sequences (12%). The sensitivity of this method, however, wasn't sufficient to detect differences in the distribution of CMV nucleic acids between the patient groups under investigation. With a very sensitive method like the polymerase chain reaction we showed that a much higher percentage (90%) of atherosclerotic patients contained CMV DNA in their arterial walls as compared to patients with maximally grade I atherosclerosis (53%). In addition, with this method we demonstrated that both immediate early and late sequences were always simultaneously present in all the reactive samples.

As already stated (5), the percentage of samples containing CMV nucleic acids as shown by dot blot hybridization with the ES probe (27%) equals the percentages found by others (1, 4) using DNA probes derived from various genomic regions. This means that all samples containing sufficient viral DNA will show hybridization reactivity using this technique, regardless of the probe used. An important conclusion that may be drawn from these findings is that the whole viral genome is present in the human arterial wall. Our finding, using dot blot hybridization techniques, that arterial wall specimen showing hybridization reactivity with the ES-probe almost always showed reactivity with the BH-probe too, further substantiates this thesis.

Using *in situ* DNA hybridization with the ES-probe we found a considerably higher percentage (50%) of the samples investigated to contain CMV nucleic acids as compared to the dot blot technique (27%). Our explanation, confirmed with RNase digestion (5), was that not only DNA-DNA hybrids formed but that also mRNA-DNA hybrids contributed significantly to the hybridization reactivity observed. These mRNAs transcribed from IE genomic regions seem to be present not only during reproductive viral cycles (6) but also during latent infections. However, using *in situ* DNA hybridization with the BH probe only a small percentage (14%) of samples could be demonstrated to contain CMV nucleic acids, suggesting that no mRNA-DNA hybrids formed. The presence of IE mRNAs has been demonstrated using *in situ* DNA hybridization techniques with DNA probes derived from IE genomic regions for Herpes Simplex virus latently present in human trigeminal ganglia (16, 17, 18), while no HSV nucleic acids could be detected when these ganglia were probed with DNA derived from genomic regions coding for structural proteins (15). Our results with both DNA hybridization techniques therefore show a parallel situation for CMV, indicating that the human arterial wall is a site of latency for this virus. Histologically, the CMV nucleic acid sequences detected with both probes were mostly located in smooth muscle cells in the media of apparently normal arterial tissue.

Using the PCR we showed that all samples reactive with primers derived from IE genomic regions were also reactive with primers derived from late genomic regions thus proving that probably the whole viral genome is conserved in the human arterial wall. The percentage of samples obtained from patients with maximally grade I atherosclerosis containing CMV nucleic acids as shown by in situ DNA hybridization with the ES probe (58%) equals the percentage found by PCR (53%). Both techniques seem to have equal sensitivity when studying apparently normal arterial walls using these probes and primers. However, when studying severely atherosclerotic arterial walls, PCR is superior. This can be explained by the localization of the CMV nucleic acids in the apparently normal smooth muscle cells in the arterial media. This arterial media has undergone massive atheromatous changes with greatly increased numbers of mononuclear leukocytes with macrophages and T-lymphocytes (19) in patients suffering from severe atherosclerosis with only few smooth muscle cells left, reducing the chance of finding CMV nucleic acids by in situ DNA hybridization. The finding that 90% of the samples obtained from patient suffering from severe atherosclerosis contained CMV nucleic acids using PCR, as compared to 53% of the samples obtained from patients with maximally grade I atherosclerosis does suggest a role for this virus in the pathogenesis of this disease. The reactivation of this latent virus to a local lytic infection in the vessel wall might result in repeated local inflammatory reactions that, in concert with other established risk factors, may lead to atheroma formation.

References

1. Melnick JL, Petrie BL, Dreesman GR, Burek J, McCollum CH, Debakey ME. Cytomegalovirus antigen within human arterial smooth muscle cells. *Lancet* 1983, 2: 644-647.
2. Petrie BL, Melnick JL, Adam E, Burek J, McCollum CH, Debakey ME. Nucleic acid sequence of cytomegalovirus in cells cultured from human arterial tissue. *J Infect Dis* 1987, 155: 158-159.
3. Benditt EA, Barret T, McDougall JK. Viruses in the etiology of atherosclerosis. *Proc Natl Acad Sci USA* 1983: 6386-6389.
4. Yamashiroga HM, Ghosh L, Yang R, Robertson L. Herpes viridae in the coronary arteries and aorta of young trauma victims. *Am J Path* 1988, 130: 71-79.
5. Hendrix MGR, Dormans PHJ, Kitslaar P, Bosman F, Bruggeman CA. The presence of CMV nucleic acids in arterial walls of atherosclerotic and non-atherosclerotic patients. *Am J Path* 1989; 134: 1151-1157.
6. Stinski MF, Thomsen DR, Stenberg RM, Goldstein LC. Organization and expression of the immediate early genes of the human cytomegalovirus. *J Virol* 1983, 46: 1-14.
7. Maniatis T, Fritsch EE, Sambrook J. *Molecular cloning - a laboratory manual*. Cold Spring Harbor Lab. Cold Spring Harbor, New York, 1982.
8. Somogyi T, Colimon R, Michelson S. An illustrated guide to the structure of the human cytomegalovirus genome and a review of transcription data. *Prog Med Virol* 1986, 33: 99-133.
9. Pande H, Baak SW, Riggs AD, Clark BR, Shively JE, Zaia JA. Cloning and physical mapping of a gene fragment coding for a 64-Kilodalton major late antigen of human cytomegalovirus. *Proc Natl Acad Sci USA* 1984, 81: 4965-4969.
10. Rüger B, Klages S, Walla B, Albrecht J, Fleckenstein B, Tomlinson P, Barrel B. Primary structure and transcription of the genes coding for two phosphoproteins pp65 and pp71 of human cytomegalovirus. *J Virol* 1987, 61: 446-453.
11. Birnboim HC, Doly J. A rapid alkaline extraction method for the isolation of

- plasmid DNA. Nucl Acid Res 1979, 7: 1513-1518.
12. Meyer H, Dreesen JCFM, van Boven CPA. Molecular cloning and restriction endonuclease mapping of rat cytomegalovirus genome. J Gen Virol 1986, 67: 1327-1342.
 13. Akrigg A, Wilkinson GWG, Oram JD. The structure of the major immediate early gene of human cytomegalovirus strain AD169. Virus Research 1985, 2: 107-121.
 14. Shibata D, Martin WJ, Appleman MD, Causey DM, Leedom JM, Arnheim NA. Detection of cytomegalovirus DNA in peripheral blood of patients infected with human immunodeficiency virus. J Infect Dis 1988, 158: 1185-1192.
 15. Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich A, Arnheim N. Enzymatic amplification of β -globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 1985, 230: 1350-1354.
 16. Stevens JG, Haar L, Porter DD, Cook ML, Wagner EK. Prominence of the Herpes Simplex virus latency associated transcript in trigeminal ganglia from seropositive humans. J Infect Dis 1988, 158: 117-123.
 17. Croen KD, Ostrove JM, Dragovic LJ, Smialek JE, Straus SE. Latent Herpes Simplex virus in human trigeminal ganglia. N Engl J Med 1987, 23: 1427-1432.
 18. Wagner EK, Devi-Rao G, Feldman LT, Dobson AT, Zhang Y, Flanagan WM, Stevens JG. Physical characterization of the Herpes Simplex virus. Latency-associated transcript in neurons. J Virol 1988, 62: 1194-1202.
 19. Hansson GK, Jonasson L, Holm J, Claesson-Wish L. Class II MHC antigen expression in the atherosclerotic plaque: smooth muscle cells express HLA-DR, HLA-DQ and the invariant gamma chain. Clin Exp Immunol 1986, 64: 261-268.

CHAPTER 5

Cytomegalovirus nucleic acid distribution within the human vascular tree

M.G.R. Hendrix
M. Daemen
C.A. Bruggeman

American Journal of Pathology 1991; 138:563-567.

Abstract

The presence of human cytomegalovirus (HCMV) nucleic acids was demonstrated in almost all major arteries of HCMV-seropositive patients by polymerase chain reaction. The amount of HCMV nucleic acids present in the arterial wall was calculated to be less than 2 viral genomic equivalents per 2500 human genomic equivalents. No relation was observed between the presence of HCMV nucleic acids in the arterial wall and the existence of atherosclerotic changes. No differences were observed in the number of viral genomic equivalents present at sites with or without atherosclerosis. Apart from a role for HCMV in the pathogenesis of atherosclerosis the presence of latent HCMV in the arterial tree provides a powerful system to describe the physical state of this latently present virus.

Introduction

Infections with human cytomegalovirus (HCMV) are common, and a prominent characteristic feature of this virus is that it persists in the host after a primary infection. Frequent reactivation from this latent state has been documented by various authors (1).

Recently, several reports suggested that the arterial wall might be a site of latency for this virus (2, 5) and it was suggested that frequent reactivation from this latent state might contribute significantly to the pathogenesis of atherosclerosis (2, 3, 4, 5, 6, 7).

In our laboratory we could demonstrate by dot blot and in situ DNA hybridization, and by polymerase chain reaction (PCR) (4, 5), that the complete viral genome was present in a latent form in a high percentage of arterial wall specimen obtained from proximal elastic arteries. We were also able to show by PCR that the prevalence of CMV nucleic acids was much higher in samples obtained from patients with grade III atherosclerosis (90%) as compared to samples obtained from matched controls with maximally grade I atherosclerosis (50%). These findings do indeed suggest a role for this virus in the pathogenesis of atherosclerosis. Most arterial samples, however, were taken from arteries prone to develop atherosclerosis. Various autopsy series clearly demonstrated that atherosclerosis is not a general disease of all the arteries but that there are several predilection sites in the human arterial tree at risk to develop lesions. These studies demonstrated a high prevalence of atherosclerotic lesions in abdominal aorta, carotid artery, common iliac artery and left anterior descending artery but showed a low prevalence in celiac and pulmonary arteries. Considering a role for CMV in the pathogenesis of atherosclerosis, one may suggest a tropism from this virus for these predilection sites or that simply more viral nucleic acids will accumulate at these arterial sites as compared to the non-atherosclerotic sites.

In this report we describe the distribution of CMV nucleic acids over the human arterial tree in a qualitative way as determined by polymerase chain reaction, and in a semi-quantitative way as determined by dot blot DNA hybridization.

Materials and methods

Tissue specimen

The subjects of this study consisted of HCMV-seropositive patients on whom autopsy was performed within 12 hours after death. Seropositivity for HCMV was confirmed by using a latex agglutination test (CMV scan Beckton & Dickinson, Oxford, UK).

Only patients with non-CMV related disease were included in this study. Patients suffering from immunosuppressive disease or undergoing immunosuppressive therapy were strictly excluded. The possibility of active CMV infection or reactivation of latently

present virus was ruled out by testing for the presence of IgM and IgA anti-CMV antibodies using a capture technique (8). At autopsy samples were taken from all major elastic arteries (Table 1 and 2) and from the portal vein.

Processing of tissue specimen

Tissue specimen for in situ DNA hybridization were formalin fixed and paraffin embedded using routine histologic procedures. For PCR and semi-quantitative dot blot DNA was extracted from the tissue specimen by proteolytic digestion and phenol-chloroform extraction followed by ethanol precipitation (4).

Microscopy

All sections were routinely stained with haematoxylin-eosin (H&E) and investigated for the presence of atherosclerosis. Only vessel walls with minimal intimal thickening and no signs of atheroma formation were considered to be free of atherosclerosis.

Oligomer synthesis

The oligomers used as primers in the PCR were synthesized on a DNA synthesizer (cyclone DNA synthesizer, Biosearch Inc., New Brunswick, NJ) by the fosamidid method. The sequences for the oligomer set reactive with the major Immediate early gene were based on the sequence described by Akrigg et al. (9) and were located in the ES fragment used as a probe for the DNA hybridization techniques. The sequences are: 5'-GGA-GAT-GTG-GAT-GGC-TTG-TA-3' (IE₁ upstream primer) and 5'-GAA-GGC-TGA-GTT-CTT-GGT-AA-3' (IE₂ downstream primer). The oligomer set reactive with the human β -globine gene has been described elsewhere (10). The sizes of the PCR amplification products of the IE and the β -globin targets are 170 and 110 base pairs respectively. The specificity and sensitivity of both primer sets have been tested extensively (5, 10).

Polymerase chain reaction

The polymerase chain reaction protocol performed on extracted cellular DNA has been described previously (5) and was performed on a prem III cycler (LEP Scientific, Buck, UK). After a total of 35 cycles of amplification 10 μ l of the reaction mixture was subjected to electrophoresis on 2% agarose gels (Seakem GTG FMC Bioproducts, Rockland, ME) containing 0.5 μ g/ml Ethidium Bromide. The amplification product was visualized by ultraviolet fluorescence. DNA samples that did not show reactivity with the CMV primers were further analysed with the primer set reactive with the β -globin gene to check on DNA preservation.

Hybridization probes

DNA hybridization was performed with the 7.0 Kb ES fragment (4, 5), an EcoR₁-SalI subdigestion of the EcoR₁-J fragment of CMV strain AD169. Controls consisted of total HCMV strain AD169 DNA, total rat cytomegalovirus (RCMV) DNA (11) and plasmid pBR328 DNA without inserts. In all DNA biotin was inserted by incorporation of biotin-11-dUTP (Bethesda Research Laboratories) using a random primed DNA labeling kit (Boehringer, Mannheim, FRG).

Semiquantitative dot blot DNA hybridization

Spotting of cellular DNA on nitrocellulose paper, hybridization conditions, and visualisation of successful hybridization was carried out as described previously (4, 5). The specificity of the biotinylated probes has been evaluated extensively (4, 5). Each arterial DNA sample was serially diluted to produce spots of 1, 0.5, 0.25, 0.12 μ g DNA respectively. Controls consisted of purified HCMV DNA and DNA extracted from non-infected human embryonic fibroblasts (HEF, Flow 2002, Flow Laboratories, McClean, VA). To

estimate the amount of viral DNA present in the arterial DNA samples spots were produced of 1, 0.5, 0.25, 0.12, 0.06, 0.03 picogram purified HCMV DNA made up to a total of 0.5 μ g DNA per spot with DNA extracted from non-infected HEF. In all experiments 0.1 pg of purified HCMV DNA could be detected equaling approximately 350 viral genomic equivalents (VGE) ($MW=1.7 \cdot 10^6$ Dalton). Estimating the total DNA content of one cell nucleus to be $6.6 \cdot 10^{11}$ Dalton, it is possible to calculate the amount of VGE present per human genomic equivalent (HGE).

In situ DNA hybridization

The in situ DNA hybridization protocol performed on 4 μ m thick tissue sections has been described previously (5). The specificity of the biotinylated probes as been evaluated extensively (5).

Statistical analysis

Statistical analysis was carried out using Fisher's exact tests. Results were considered significant when $p < 0.01$.

Results

Tissue specimen

Nine autopsy patients (5 men, 4 women) with a mean age of 74 years (range 47 to 92 years) were available. Three men and 4 women died of metastatic neoplastic disease (colon carcinoma: 3; breast carcinoma: 3; thyroid carcinoma: 1) and 2 men died of cardiac arrest. All subjects available for this study were shown to contain anti-HCMV antibodies of the IgG class, but not of the IgM or IgA class, which excluded primary and frequently reactivating disease. These nine autopsy patients provided 77 arterial samples.

Microscopy

From all arterial samples 58 of 77 (75%) showed clear signs of atherosclerosis with marked intimal thickening and sometimes atheroma formation. Only the pulmonal artery was almost always free of atherosclerotic changes (Table 1). Only in 1 of 7 (14%), slight intimal thickening was observed. Each individual patient showed atherosclerotic disease of 6 or more main vessels but also had arteries free of intimal thickening, making this group of patients suitable for this study.

Polymerase chain reaction

The polymerase chain reaction (PCR) performed on extracted DNA from all major arteries (Table 1) showed that 72 of 77 (94%) samples contained HCMV nucleic acids. These nucleic acids were equally distributed over the vascular tree.

There was no difference observed in the prevalence of HCMV DNA in samples obtained from arteries with atherosclerotic changes as compared with samples obtained from microscopically normal arterial walls ($p=0.36$).

Samples negative for HCMV nucleic acids by PCR (5 of 77 (6%)) were checked for DNA integrity with a primer set reactive with the human β -globin gene. All samples tested with this primer set were positive for the presence of the β -globin gene proving that the DNA integrity was sufficient for PCR analysis.

Table 1. Polymerase chain reaction and stratification for atherosclerosis.

Vessel	PCR(total) pos/total (%)	atherosclerosis pos/total (%)	PCR + and atherosclerosis +	PCR + and atherosclerosis -
Thoracic aorta	8/9	6/9	5/6	3/3
Abdominal aorta	8/9	8/9	8/8	0/1
Carotid artery	8/9	7/9	7/7	1/2
Superior mesenteric artery	8/9	8/9	7/8	1/1
Celiac artery	7/7	6/7	6/6	1/1
Renal artery	9/9	7/9	7/7	2/2
Common iliac artery	9/9	6/9	6/6	3/3
Pulmonary artery	7/7	1/7	1/1	6/6
Left anterior descending artery	8/9	9/9	8/9	0/0
Portal vein	8/8	NR	NR	NR
Total arteries (except portal vein)	72/77 (94%)	58/77 (75%)	55/58 (95%)	17/19 (90%)

NR, not relevant

Semiquantitative dot blot DNA hybridization

Using the dot blot DNA hybridization technique (Table 2), HCMV nucleic acid sequences were detected in 58 of 77 (75%) samples. HCMV DNA was detected in 43 of 58 (73%) samples obtained from atherosclerotic arteries and in 15 of 18 (83%) samples obtained from normal arteries. Statistical analysis showed this difference not to be significant ($p=0.46$). Most of the samples containing detectable amounts of HCMV nucleic acids only showed positive hybridization in the less diluted DNA samples meaning that 0.1-0.2 pg of viral DNA was present in 1 μ g DNA extracted from arterial walls. From this we concluded that approximately 1 to 2 viral genomic equivalents (VGE) were present per 2500 human genomic equivalents (HGE). Only 4 samples, all obtained from one patient, could be diluted fourfold and still show positive hybridization. This means that in these samples approximately 1 to 2 VGE were present per 600 HGE. There was no difference observed in the number of VGE per HGE present in arteries with or without atherosclerotic changes.

All samples negative with the dot blot DNA hybridization technique but positive by PCR should contain less than 1 VGE per 2500 HGE. In situ DNA hybridization of sections of arteries with atherosclerotic changes showed that 24 of 58 (41%) contained HCMV nucleic acid sequences whereas sections of arteries without these changes showed the presence of nucleic acids in 7 of 19 (37%), which was not statistically different.

In situ DNA hybridization

Histologically the HCMV nucleic acid sequences detected with the in situ DNA hybridization technique were mostly located in apparently normal arterial tissues without atherosclerotic changes. Only in a few occasions the viral nucleic acids were located in the thickened intimal layer. No HCMV nucleic acid sequences were detected in sites of atheroma formation. Sequential sections stained with H&E showed that the DNA hybridization reactivity mostly was located in smooth muscle cells in the arterial media.

Table 2. Dot blot DNA hybridization and stratification for atherosclerosis.

Vessel	Dot blot (total) pos/total (%)	atherosclerosis pos/total (%)	Dot blot + and atherosclerosis +	Dot blot + and atherosclerosis -
Thoracic aorta	8/9	6/9	5/6	3/3
Abdominal aorta	4/9	8/9	4/8	0/1
Carotid artery	5/9	7/9	4/7	1/2
Superior mesenteric artery	7/9	8/9	6/8	1/1
Celiac artery	5/7	6/7	4/6	1/1
Renal artery	9/9	7/9	7/7	2/2
Common iliac artery	7/9	6/9	5/6	3/3
Pulmonary artery	5/7	1/7	0/1	5/6
Left anterior descending artery	8/9	9/9	8/9	0/0
Portal vein	6/8	NR	NR	NR
Total arteries (except portal vein)	58/77 (75%)	58/77 (75%)	43/58 (73%)	15/19 (83%)

NR, not relevant

Discussion

In this study we have demonstrated by PCR that probably all major arteries obtained from HCMV seropositive patients contain HCMV nucleic acid sequences. These nucleic acids are equally distributed among arteries with and without atherosclerotic changes. By using semiquantitative dot blot DNA hybridization techniques it was possible to estimate that approximately 2 or less viral genomic equivalents (VGE) are present per 2500 human genomic equivalents (HGE).

There were no differences observed in the number of VGE per HGE present in arteries with or without atherosclerotic changes. HCMV nucleic acids were mostly located in smooth muscle cells of the arterial media as shown by in situ DNA hybridization.

One of the objects of this study was to look if HCMV nucleic acids preferentially accumulated at arterial sites prone to develop atherosclerotic lesions. The finding that almost all arteries contain HCMV nucleic acids and that there is no correlation with atherosclerotic sites clearly shows that there is no tropism from the HCMV for these predelection sites. These results could more or less be predicted from previous reports demonstrating the presence of HCMV in various arteries from patients with severe atherosclerosis but also in abdominal aortas without atherosclerotic changes (2, 3, 4, 6). Also from our previous findings using PCR (5) and demonstrating a high prevalence of HCMV nucleic acids in patients with grade III atherosclerosis (90%) but also in patients with maximally grade I atherosclerosis (50%) did suggest these results. As already stated (4, 5) we don't suggest a direct role for HCMV in the pathogenesis of atherosclerosis.

The finding that arteries with or without atherosclerotic changes contain approximately equal numbers of viral genomic equivalents (VGE) per human genomic equivalent (HGE) also means that there is no direct relationship between the HCMV and the presence of atherosclerosis and again a possible reason for these findings is that the vessels under investigation represent end stage atherosclerotic changes not influenced anymore by the HCMV. The role for HCMV in the pathogenesis of atherosclerosis might be at the start of this disease with the development of fatty streaks (12, 13). Considering the tropism from HCMV for endothelial cells (15) infection of these cells could result in the production of inflammatory mediators such as interleukin I, which subsequently induce activation and adherence of leucocytes responsible for injury of the endothelium (13) being the initial process in the response to injury theory of Ross (14). This injury, in

co-operation with other established risk factors, could start the process of atherogenesis. Once present in the arterial wall the HCMV will remain latently present for the rest of life. Transient local reactivation from this latent state (16) might also contribute further to the process of atherogenesis.

Apart from the process of atherogenesis the presence of latent HCMV in the human arterial wall remains an intriguing phenomenon. Considering the wide tropism of this virus for many different tissues during reproductive infection (17) one might ask if the human vascular wall is the only site of latency. Probably more celltypes will contain latent HCMV. Our finding that at most 2 VGE are present per 2500 HGE may substantiate this probability because these low numbers were until recently beyond the reach of molecular biology.

Comparable viruses like Herpes Simplex virus (HSV) were found to be present at at least 1 VGE per 100 HGE in trigeminal ganglia (18) facilitating their detection. Objects for further research are therefore obvious. At first screening other tissues on the presence of latent HCMV and secondly characterization of the physical state of this virus at least latently present in the human arterial tree.

References

1. Ho, M. Cytomegalovirus biology and infection. Plenum Medical Book Co. 1982, New York.
2. Melnick, J.L., Petrie, B.L., Dreesman, G.R., Burek, J., McCollum, C.H., Debakey, M.E. Cytomegalovirus antigen within human arterial smooth muscle cells. *Lancet* 1983; 2: 644-647.
3. Yamashiroga, H.M., Ghosh, L., Yang, R., Robertson, L. Herpes viridae in the coronary arteries and aorta of young trauma victims. *Am. J. Path.* 1988; 130: 71-79.
4. Hendrix, M.G.R., Dormans, P.H.J., Kitslaar, P., Bosman, F., Bruggeman, C.A. The presence of CMV nucleic acids in arterial walls of atherosclerotic and non-atherosclerotic patients. *Am. J. Path.* 1989; 134: 1151-1157.
5. Hendrix, M.G.R., Salimans, M.M.M., van Boven, C.P.A., Bruggeman, C.A. High prevalence of latently present cytomegalovirus in arterial walls of patients suffering from grade III atherosclerosis. *Am. J. Path.* 1990; 136: 23-28.
6. Melnick, J.L., Adam, E., Debakey, M.E. Possible role of cytomegalovirus in atherogenesis. *J.A.M.A.* 1990; 263: 2204-2207.
7. Grattan, M.T., Moreno-Cabral, C.E., Starnes, V.A., Oyer, P.E., Stinson, E.B., Shumway, N.E. Cytomegalovirus infection is associated with cardiac allograft rejection and atherosclerosis. *J.A.M.A.* 1989; 261: 3361-3366.
8. van Loon, A.M., Heessen, F.W.A., van der Logt, J.Th.M., van der Veen, J. Direct enzyme-linked-immunosorbent-assay that uses peroxidase-labeled antigen for determination of immunoglobulin M antibody to cytomegalovirus. *J. Clin. Micr.* 1981; 13: 416-422.
9. Akrigg, A., Wilkinson, G.W.G., Oram, J.D. The structure of the major immediate early gene of human cytomegalovirus strain AD169. *Virus Research* 1985; 2: 107-121.
10. Saiki, R.K., Schaaf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, A., Arnheim, N. Enzymatic amplification of β -globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 1985; 230: 1350-1354.
11. Meijer, H., Dreesen, J.C.F.M., van Boven, C.P.A. Molecular cloning and restriction endonuclease mapping of the rat cytomegalovirus genome. *J. Gen. Virol.* 1986; 67: 1327-1342.
12. Munro, M.J., Cotran, R.S. The pathogenesis of atherosclerosis: atherogenesis

- and inflammation. *Lab. Invest.* 1988; 58: 249-261.
13. Bruggeman, C.A., van Dam-Mieras, M.C.E. The possible role of Cytomegalovirus in atherogenesis. *Progress in Medical Virology* 1990, Karger AG, Basel, Switzerland. (in press).
 14. Ross, R. The pathogenesis of atherosclerosis: an update. *New Engl. J. Med.* 1986; 314: 488-500.
 15. Toorkey, C.B., Carrigan, D.R. Immunohistochemical detection of an immediate early antigen of human cytomegalovirus in normal tissues. *J. Infect. Dis.* 1989; 160: 741-751.
 16. McVoy, Adler. Immunologic evidence for frequent age-related CMV reactivation in seropositive immunocompetent individuals. *J. Infect. Dis.* 1989; 160: 1-10.
 17. Myerson, D., Hackman, R.C., Nelson, J.A., Ward, D.C., McDougall, J.K. Widespread presence of histologically occult cytomegalovirus. *Human Path.* 1984; 15: 430-439.
 18. Efsthion, S., Minson, A.C., Field, H.J., Anderson, J.R., Wildy, P. Detection of Herpes Simplex virus-specific DNA sequences in latently infected mice and in humans. *J. Virol.* 1986; 57: 446-455.

CHAPTER 6

Widespread presence of cytomegalovirus DNA in tissues of healthy trauma victims

Ron M.G. Hendrix
Mariette Wagenaar
Rob L. Slobbe
Catrien A. Bruggeman

Journal of Clinical Pathology 1997; 50:59-63

Abstract

Aims: To determine the localisation of human cytomegalovirus (HCMV) DNA in abdominal aorta, spleen, and transplantable organs such as kidney, pancreas, and liver obtained from healthy individuals; to characterise the cell type(s) in these tissues that serve as a reservoir for latent HCMV.

Methods: HCMV DNA was detected by dot blot DNA hybridization and in situ DNA hybridization with a probe for CMV major immediate early sequences (UL123) and nested PCR with primers derived from the CMV major immediate early (IE) gene exon 4 (UL123ex4). Samples of liver, abdominal aorta, spleen, kidney and pancreas were obtained at necropsy or at donor nephrectomy from healthy patients.

Results: CMV DNA was detected in most tissue samples using dot blot hybridization and nested PCR. In situ hybridization demonstrated that, in addition to smooth muscle cells in the arterial wall, hepatocytes, tubular and glomerular kidney cells, splenic red pulp cells, and pancreatic acinar cells also harboured CMV DNA. CMV DNA was detected in seropositive and in some seronegative subjects.

Conclusion: CMV DNA is widely distributed in organs of healthy subjects. CMV DNA was found in various cell types in several organs, suggesting that during latency, CMV DNA is present throughout the body.

Introduction

Infection by human cytomegalovirus (CMV), a herpesvirus, can result in a variety of disorders ranging from subclinical disease in normal subjects to often fatal disease in immunocompromised patients (1). CMV can establish latent infections. Virus reactivation followed by active infection has been clearly demonstrated in recipients of organ transplants and often leads to life threatening disease (1). Seronegative recipients of donor organs are at particular risk of developing CMV disease (1). Molecular epidemiological studies have shown that two recipients receiving organs from the same donor excrete identical CMV strains in the urine (2), demonstrating that HCMV transmission occurs with the graft. However, the cell types or types of graft that serve as a reservoir for latent CMV are unknown. Several reports have suggested that latent CMV is present in the smooth muscle cells of the arterial wall (3, 4, 5, 6). Using PCR, we detected CMV DNA in the arterial wall in almost 70% of CMV seropositive patients (5) and using in situ DNA hybridization showed that arterial smooth muscle cells harboured the virus. In other reports CMV nucleic acids were found in peripheral leucocytes obtained from healthy CMV seropositive and seronegative subjects (7, 8).

In this report we describe the presence and location of HCMV DNA in aorta, kidney, spleen and pancreas of healthy organ donors.

Materials and methods

Tissue specimen

Two groups of patients were included in this study.

Group 1 comprised trauma victims (eight men, five women) available for donor nephrectomy. Samples were taken from the abdominal aorta, spleen and pancreas using strict aseptic surgical techniques. Twelve artery, 12 spleen, and 8 pancreatic samples were collected from these subjects. The mean age of the subjects was 35 years (range 8 to 45 years). CMV DNA was detected using in situ DNA hybridization and PCR. If there was sufficient tissue, dot blot DNA hybridization was also done.

Group 2 comprised patients (six men, two women) with no relevant medical history who died of sudden cardiac arrest. Samples were taken at necropsy from the spleen, liver, and kidney. All samples were taken within 12 hours of death using aseptic techniques. Eight spleen, 8 liver, and 8 kidney samples were collected. The mean age of the subjects was 65 years (range 54 to 75 years). In most cases CMV was detected by PCR only. Some liver and kidney samples were examined using in situ DNA hybridization to identify the cell types harbouring CMV DNA.

All tissue samples were handled separately to avoid cross contamination. Tissues from which DNA was to be isolated were stored at 4° C in 50% ethanol. Tissues for in situ DNA hybridization were fixed routinely in formalin and embedded in paraffin wax.

Serology

Seropositivity for human Cytomegalovirus was determined for all patients using a latex agglutination test (CMV-scan, Becton Dickinson, Cockeysville, MD, USA) (9), an IgG ELISA from Organon Teknika, Boxtel, The Netherlands, and an in-house ELISA based on AD169 infected cell lysate. To establish active infection, IgM anti-HCMV antibodies were detected using a capture ELISA technique (10). All serum samples were taken directly on arrival of the subjects at our hospital.

DNA extraction from tissues and tissue sections

DNA for dot-blot DNA hybridization and PCR analysis was extracted from the ethanol preserved tissues. Small tissue samples were collected in 1.5 ml polypropylene microcentrifuge tubes. Subsequent proteolytic digestion of all samples was carried out overnight at 50° C in a mixture of 10 mM Tris-HCl (pH=7.5), 5 mM EDTA, 100 mM NaCl, 250 microgram/ml proteinase K (Boehringer Mannheim, Federal Republic of Germany) and 10 mg/ml SDS under constant agitation. The DNA was purified by phenol-chloroform extraction and ethanol precipitation and finally dissolved in 50 microliter distilled water. To exclude the possibility of cross contamination during these procedures, herring sperm DNA was included as a negative control. DNA were included.

Oligonucleotides

Oligonucleotides purchased from Eurogentec (Liege, Belgium) were derived from the CMV major immediate early gene exon 4 (UL123ex4). The nucleotide sequences of primers used for nested PCR are: first round: A1 5'-ACGAA-TTCGT-AATGA-AGCGC-CGCAT-TGA-3' and A2 5'-ACGAG-CTGCA-ATAGT-GACGT-GGGAT-CCA-3'; second round: B1 5'-ACGAT-TTCTC-TGCAT-GAAGG-TCTTT-GC-3' and B2 5'-ACGAG-CTCGT-GGGAT-CCATA-ACAGT-AA-3'; internal probe: C 5'-CAGTA-ATTGT-GGCGT-AGAAC-AGTGA-TCAGG-3'. The nested PCR product was 468 basepairs. The primer set reactive with the B-globin gene has been described previously (11).

Hybridization probes

A 7.0 kb EcoR1-Sal1 fragment from the EcoR1-J fragment of the CMV AD169 strain (5) which contains the major immediate early gene, was used as a probe. The probe was labelled with biotin by incorporating biotin-11-dUTP (Bethesda Research Laboratories, U.S.A.), using a random primed DNA labelling kit (Boehringer Mannheim, Germany).

Dot blot DNA hybridization

Transfer of cellular DNA to nitrocellulose paper, hybridization conditions, and visualisation of successful hybridization were carried out as described previously (5). The specificity of the biotinylated probe has been evaluated extensively (5).

Nested polymerase chain reaction

The reaction mixture contained standard PCR buffer (10x PCR buffer, Promega, USA)

supplemented with MgCl_2 to a final concentration of 2.4 mM and 250 μM of each dNTP in a reaction volume of 50 μl . One nanogram of each primer and 1 unit DNA polymerase (Ampli-taq, Perkin-Elmer-Cetus, USA) and 200 ng target DNA were added. After 30 cycles of 1 minute at 94 C, 1 minute at 58 C and 2 minutes at 72° C on a Prem III thermal cycler (LEP Scientific, Great Britain), 1 μl of the reaction mixture was transferred to the second reaction tube containing the nested primer for the second round PCR, carried out under the same conditions. After 40 cycles, 10 μl of the second reaction mixture was subjected to electrophoresis on 2% agarose gels (Seakem GTG FMC Bioproducts, USA) and transferred to a nylon filter (Hybond N+ Amersham, Great Britain). The internal oligonucleotide was end-labelled with ^{32}P and used as a probe for hybridization of the nylon filters. All samples showing hybridization were considered to contain CMV DNA.

In situ DNA hybridization

The presence of CMV nucleic acids was detected using in situ DNA hybridization on sections 4 μm thick as described previously (5).

Immunohistochemistry

CMV specific antigens were detected immunohistochemically as described previously (12). A monoclonal antibody directed against the CMV 72 kilodalton IE antigen (UL123) (Dupont speciality diagnostics, Wilmington, USA) and the MC222 monoclonal antibody directed against the 65 kilodalton tegument protein of the virus particle, pp65 (ppUL8-3(PK)), were used (13)

Results

Serology.

Serum samples from four group I subjects did not contain CMV antibodies. All other subjects were CMV- positive. Results of all IgG tests showed 100% agreement. No CMV reactive antibodies of the IgM class were detected indicating that none of the subjects had active CMV disease.

Immunohistochemistry

Immunohistochemical studies were done using both monoclonal antibodies. Immunoreactivity was not observed in any of the samples tested

Dot blot DNA hybridization

Twenty-three tissue samples from group 1 subjects were subjected to dot blot hybridization. Overall, 6 of 7 samples of aorta, 10 of 10 samples of spleen, and 3 of 6 samples of pancreas harboured CMV DNA (table 1). Tissue samples from three of four seronegative donors (2 aorta, 3 spleen, and 1 pancreas) were evaluated by this technique. Of these, samples of aorta and spleen contained viral DNA, while that of the pancreas did not.

Polymerase chain reaction

In group 1 (table 1), 9 of 13 abdominal aorta, 12 of 12 spleen, and 6 of 8 pancreatic samples contained viral DNA. CMV DNA was also found in 3 of 4 seronegative subjects. In group 2, 8 of 8 spleen, 8 of 8 liver and 8 of 8 kidney samples harboured CMV DNA. The few samples which did not react with the CMV primers reacted well with the beta-globin primers. None of the numerous negative controls included nor any of the samples containing DNA from the other known herpesviruses were positive.

Table 1 Results of the dot-blot DNA hybridization, in situ DNA hybridization, and PCR performed on tissue specimens obtained from previously healthy heart-beating donors. Each column represents a subject.

	p1	p2	p3	p4	p5	p6	p7	p8	p9	p10	p11	p12	p13	total
dot-blot (ES)	aorta	+	+	NA	+	NA	-	+	+	NA	NA	+	NA	6/7
	spleen	+	+	+	+	NA	+	+	+	NA	NA	+	+	10/10
	pancreas	NA	+	-	-	NA	-	NA	NA	NA	NA	NA	+	3/6
In-situ (ES)	aorta	+	+	+	+	-	-	+	-	-	-	+	NA	6/12
	spleen	+	+	+	+	NA	+	-	+	-	-	+	+	8/12
	pancreas	NA	+	+	+	NA	-	NA	+	NA	+	NA	+	6/7
PCR (MIE-4)	aorta	+	+	+	+	-	-	+	+	-	+	+	NA	9/12
	spleen	+	+	+	+	NA	+	+	+	+	+	+	+	12/12
	pancreas	NA	+	+	+	NA	-	NA	+	+	+	NA	+	6/8
serology		-	+	-	+	-	+	-	+	+	+	+	+	4/13

Fig. 1 Photomicrographs of in situ DNA hybridization of samples from four organs, using HCMV-specific probes. The dark spots (arrow) represent hybridization. All sections are counterstained with methyl green. Panel A: Kidney (400x), Panel B: spleen (400x), Panel C: liver (400x), Panel D: pancreas (400x).

In situ DNA hybridization

The red pulp of the spleen often contained HCMV positive cells (fig. 1). In the kidney the epithelial cells of the tubulus were positive frequently. CMV DNA was also detected in epithelial cells of the glomerulus (fig. 1). Pancreatic acinar cells (fig. 1) hepatocytes were often positive.

Discussion

We have shown in the present study, that potential donor organs from both seropositive and seronegative individuals can contain CMV DNA.

All Subjects were anti-CMV IgM negative, excluding active CMV infection. None of the subjects had had an immunosuppressive disease, again allowing us to conclude that they did not have an active CMV infection. Therefore, the CMV DNA detected probably represents latent and not active virus.

As the hybridization studies do not discriminate between latency and low level infection we used immunohistochemistry to detect viral gene products. We did not observe immunoreactivity with a monoclonal antibody directed against immediate early gene products (UL123) nor with a monoclonal antibody directed against a late gene product, the viral tegument protein pp65 (ppUL83(pk)). These findings suggest that latent CMV and not a low level productive infection was detected. Interestingly, Toorkey et al (14) detected CMV immediate early gene products, but not early nor late gene products, in normal tissue suggesting latent rather than low level productive infection. However, this immunoreactivity with immediate early gene products was observed only with a single batch of the monoclonal antibody and could not be reproduced with another batch of that same antibody.

Our findings that CMV DNA can be detected in the aorta, spleen, pancreas, kidney, and liver suggest that the human arterial wall and peripheral leucocytes are not the only reservoirs for latent CMV as described previously (5, 6, 7, 8), but that a variety of organs can harbour the viral genome.

PCR is very sensitive to cross contamination between specimens (15) and to contamination by previously amplified DNA (15). However, the overall agreement of results obtained by dot blot hybridization, in situ hybridization, and PCR, and the absence of viral DNA in the numerous negative controls included in the PCR experiments in this study preclude the possibility of cross-contamination.

PCR studies of murine cytomegalovirus (MCMV) have shown that the lung, spleen, brain, heart, kidney, liver and salivary glands harbour latent MCMV DNA (16, 17, 18, 19). The exact nature of the cell(s) carrying this MCMV DNA remain obscure. As shown in the present study by in situ DNA hybridization, many different cell types, such as hepatocytes, tubular and glomerular cells, and splenic red pulp cells, contained viral DNA. During active infection the virus is distributed widely and replicates in virtually all cell types as shown by the appearance of specific viral cytopathological changes, the presence of viral antigens and the presence of viral DNA in various cells in almost all organs (20, 21).

Our finding that CMV DNA is present in a variety of organs obtained from previously healthy seropositive subjects was not surprising, as several studies have clearly demonstrated transmission of CMV present in transplanted organs and peripheral blood obtained from CMV seropositive donors (22). The observation that in this study three of four seronegative patients carried CMV DNA is in accordance with other reports describing the presence of CMV nucleic acids in leucocytes (7, 8, 23, 24) and tissues (25, 26) obtained from seronegative patients. Whether this HCMV DNA represents latent virus or defective virus remains unknown.

In summary: a variety of organs such as spleen, kidney, liver and pancreas contain

CMV DNA as demonstrated by PCR and dot blot hybridization. The presence of CMV DNA was demonstrated in seropositive and also in seronegative subjects by dot blot DNA hybridization, in situ DNA hybridization and nested PCR. Arterial smooth muscle cells, hepatocytes, tubular and glomerular kidney cells, splenic red pulp cells and pancreatic acinar cells, contain HCMV DNA. The absence of viral gene products in these cells suggests latent rather than low level active infection.

References

1. Ho M. Cytomegalovirus biology and infectium. New York; Plenum Medical Book Co., 1982.
2. Chou S. Acquisition of donor strains of cytomegalovirus by renal transplant recipients. *N Engl J Med* 1986;314:1418
3. Melnick JL, Petrie BL, Dreesman GR, Burek J, McCollum CH, Debakey ME. Cytomegalovirus antigen within human arterial smooth muscle cells. *Lancet*; 1983,ii:644-647.
4. Yamashiroya HM, Ghosh L, Yang R, Robertson L. Herpes viridae in the coronary arteries and aorta of young trauma victims. *Am J Pathol* 1988;130:71-79.
5. Hendrix MGR, Salimans MMM, van Boven CPA, Bruggeman CA. High prevalence of latently present Cytomegalovirus in arterial walls of patients suffering from grade III atherosclerosis. *Am J Pathol* 1990;136:23-28.
6. Hendrix MGR, Daemen M, Bruggeman CA. Cytomegalovirus nucleic acid distribution within the human vascular tree. *Am J Pathol* 1991;138:563-567.
7. Bevan IS, Daw RA, Day PJR, Ala FA, Walker MR. Polymerase chain reaction for detection of human cytomegalovirus infection in a blood donor population. *Br J Haematol* 1991;78:94-99
8. Taylor-Wiedeman J, Hayhurst GP, Sissons JGP, Sinclair JH. Polymorphonuclear cells are not sites of persistence of human cytomegalovirus in healthy individuals. *J Gen Virol* 1993;74:265-268
9. Kraat YJ, Hendrix MGR, Landini MP, Bruggeman CA. Comparison of four techniques for detection of antibodies to CMV. *J Clin Microbiol* 1992;30:522-524.
10. van Loon AM, Heessen FWA, van der Logt JThM, van der Veen J. Direct enzyme linked immunosorbent assay that uses peroxidase-labeled antigen for determination of IgM antibody to cytomegalovirus. *J Clin Microbiol* 1981;13:416-422.
11. Saiki RK, Schauf S, Faloona F, Mullis KB, Horn GT, Ehrlich A, Arnheim N. Enzymatic amplification of B-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 1985;230:1350-1354.
12. Hendrix MGR, Dorman PHJ, Kitslaar P, Bosman F, Bruggeman CA. The presence of CMV nucleic acids on arterial walls of atherosclerotic and non-atherosclerotic patients. *Am.J.Pathol.* 1989;134:1151-1157.
13. Lijnschoten G, Stals F, Bruggeman CA, Havenith MH, Geraedts JPM. The presence of cytomegalovirus antigens in karyotyped abortions. *Am.J.Reprod.Immunol.* 1994;32:211-20.
14. Toorkey CB, Carrigan DR. Immunohistochemical detection of an immediate early antigen of human cytomegalovirus in normal tissues. *J.Infect.Dis.* 1989;160:741-751.
15. Persing DH. Polymerase chain reaction: trenches to benches. *J Clin Microbiol* 1990;29:1281-1285
16. Klotman ME, Henry SC, Greene RC, Brazy PC, Klotman ME, Hamilton JD. Detection of mouse cytomegalovirus nucleic acid in latently infected mice by in vitro enzymatic amplification. *J Infect Dis* 1990;161:220-225
17. Balthesen M, Messerle M, Reddehase MJ. Lungs are a major organ site of

- cytomegalovirus latency and recurrence. *J. Virol.* 1993;67:5360-5366
18. Collins T, Pomeroy C, Jordan MC. Detection of latent cytomegalovirus DNA in diverse organs of mice. *J. I.D.* 1993;168:725-9
 19. Yuhasz SA, Dissette VB, Cook ML, Stevens JG. Murine cytomegalovirus is present in both chronic active and latent states in persistently infected mice. *Virology* 1994;202:272-280
 20. Myerson P, Hackman RC, Nelson JA, Ward DC, McDougal JK. Widespread presence of histologically occult cytomegalovirus. *Hum Pathol* 1984; 15:430-439.
 21. Wu T-C, Lee WA, Pizzorno MC, Au W-C, Chan Y-J et al. Localization of the human CMV 2.7-kb major early B-gene transcript by RNA in situ hybridization in permissive and nonpermissive infections. *Am J Pathol* 1992;141:1247-1254.
 22. Ho M. Epidemiology of Cytomegalovirus infections. *Rev Infect Dis* 1990;12:S701-S710
 23. Stanier P, Taylor DL, Kitchen AD, Wales N, Tryhorn Y, Tyms AT. Persistence of cytomegalovirus in mononuclear cells in peripheral blood from blood donors. *Br Med J* 1989;299:897-898
 24. Zang et al. Detection of HCMV DNA-RNA and antibody in normal blood donors. *J Infect Dis* 1995;171:1002-1006
 25. Kraat YJ, Hendrix MGR, Wijnen RMH, et al. Detection of latent human cytomegalovirus in organ tissue and the correlation with serological status. *Transplant Int* 1992;5(suppl 1):S613-S616
 26. Schonlan U, Crombach M, Maisch B. Assessment of cytomegalovirus DNA and protein expression in patients with myocarditis. *Clin Immunol Immunopathol* 1993;68:229-233

CHAPTER 7

Detection and sequence analysis of the major immediate early and PP150 gene of latent human cytomegalovirus in spleen, liver and kidney tissues of trauma victims

Ron M.G. Hendrix
Eric Beuken
Rob L. Slobbe
Catrien A. Bruggeman

Journal of Medical Virology 1996; 50:193-197.

Abstract

The presence of human cytomegalovirus (HCMV) DNA in liver, spleen and kidney samples of HCMV seropositive trauma victims during latency was demonstrated by PCR, using primers reactive with the major immediate early gene exon 4 and the structural gene pp150. Sequence analysis of the PCR amplicates showed more than 95% homology with the reference HCMV strain AD169. The few mutations observed were mostly distributed randomly. In one subject 2 types of the MIE-4 gene were detected, and in another subject two types of the pp150 gene were found, suggesting that different strains of HCMV can be found in organs of the same patient during latency.

Introduction

In general, human cytomegalovirus (HCMV), a member of the Herpesviridae, infects immunocompetent individuals without causing clinical disease (1). The prevalence of antibodies to HCMV varies from 40 to 80%, depending on the person's socioeconomic status (2). After primary infection, the virus becomes latent but can be reactivated during periods of immunosuppression, and this can lead to life threatening disease (1). The cell type or types that act as a reservoir for the latent HCMV, however, remain(s) obscure. Polymerase chain reaction (PCR) analysis has shown that both peripheral leucocytes (3, 4, 5, 6) and vascular smooth muscle cells (7, 8, 9, 10), can harbor latent HCMV. The data concerning leukocytes are difficult to interpret because several reports demonstrate that HCMV DNA is present not only in seropositive healthy individuals, but also in those who are seronegative (3, 4, 5, 6), whereas in other reports no HCMV DNA could be detected (11). There were great differences in DNA preparation procedures and PCR reaction conditions between these reports, which may contribute to the differences observed. Furthermore, in none of these reports were the sequence of the PCR product analyzed, its identity only being confirmed by hybridization with end-labeled oligonucleotide as probe. Significant sequence variation, as described for clinical HCMV isolates within the gB gene (12, 13), the LS junction (14), and the major immediate early gene (UL123EX4) (15) would therefore go undetected. Sequence analysis can help control an inherent problem of PCR, which is its susceptibility to artifacts caused by mispriming with genomic DNA, or with DNA from other Herpesviridae. However, PCR is a powerful tool for elucidating epidemiological patterns in the pathogenesis of HCMV (12, 13, 14).

Blood and/or blood products are mostly depleted of leukocytes, and therefore not a major route of HCMV transmission (16), but organ transplantation (1), especially from seropositive donors to seronegative recipients, is a potential route of infection. It therefore seems prudent not only to examine organs available for transplantation such as the liver and kidney, but also other available organs such as the spleen, for the presence of latent HCMV, employing the same techniques used for leukocyte analysis.

In this study, we describe the presence of HCMV nucleic acids, as detected by nested PCR in specimen of spleen, kidney, and liver obtained from previously healthy trauma victims. We also present sequence data for the PCR products, proving that it is indeed HCMV which is detected.

Materials and methods

Tissue specimen

Specimen of spleen, kidney, and liver from previously healthy trauma victims were collected at autopsy, using strict aseptic techniques. All specimens were kept apart and

were directly placed, in the autopsy room, in 70% ethanol-phosphate-buffered saline and stored at 4°C until use. Autopsy was performed within 12 hours of death. None of the trauma victims had received blood and/or blood products. Eight patients who died of major brain trauma (two females, six males) provided seven spleen samples, eight kidney samples and six liver samples. The mean age of the subjects was 38 years (range 18 to 45 years).

Serology

Seropositivity for HCMV was determined for all subjects, using a latex agglutination test (CMV-scan, Becton & Dickinson, Great Britain) which detects antibodies of both the IgG and IgM class (17), and an ELISA (Organon-Teknika, The Netherlands). All sera were collected directly on arrival of the subjects at the hospital.

DNA extraction from tissues

DNA for PCR analysis was extracted from the ethanol preserved tissues. Small tissue samples were collected in 1.5 ml polypropylene microcentrifuge tubes. Proteolytic digestion of all samples was carried out overnight at 50°C in a mixture of 10 mM Tris-HCl (pH=7.5), 5 mM EDTA, 100 mM NaCl, 250 µg/ml proteinase K (Boehringer Mannheim, Germany) and 10 mg/ml sodium dodecylsulphate under constant agitation. The DNA was purified by phenol-chloroform extraction and ethanol precipitation and was finally dissolved in 50 µl distilled water. The DNA was quantified spectrophotometrically. To evaluate the possibility of cross-contamination with HCMV DNA during this procedure, every second sample served as a negative control containing herring sperm DNA only and was processed in the same way as the tissue samples.

Oligomer synthesis

Oligomers were purchased from Eurogentec (Liege, Belgium). One nested set was derived from the HCMV major immediate early gene exon 4 (UL123EX4). The nucleotide sequences of the primers used were: first reaction: A1 5'-ACGAA-TTCGT-AATGA-AGCGC-CGCAT-TGA-3' and A2 5'-ACGAG-CTGCA-ATAGT-GACGT-GGGAT-CCA-3'; nested reaction: B1 5'-ACGAT-TTCTC-TGCAT-GAAGG-TCTTT-GC-3' and B2 5'-ACGAG-CTCGT-GGGAT-CCATA-ACAGT-AA-3'. The nested PCR product was 468 basepairs long. A second nested set was derived from the large structural phosphoprotein pp150 (ppUL32(bpp)) (19). The nucleotide sequences of the primers used were: first reaction: C1 5'-GGTGC-TGTTC-AACGA-GCTCA-3' and C2 5'-GGTCC-AGGTT-AAGGT-CGAGC-3'; nested reaction: D1 5'-TTGGG-ATACT-ACCGC-GAGCT-3' and D2 5'-TCACG-CAGAA-CATGT-TACGG-3'. The nested product was 360 basepairs long. No cloning experiments or other tests had been performed in our laboratory with DNA coding for pp150 prior to these PCR experiments. All primers were tested for reactivity with DNA sequences stored in the EMBL genebank database and were demonstrated to match stored HCMV sequences only.

Nested polymerase chain reaction

All PCR experiments were performed under identical reaction conditions. Primer sets were used in separate reactions, but also in a duplex set-up. The first reaction was performed in 50 µl, under standard conditions (9).

After 30 cycles (melting: 1 min at 94°C; annealing: 1 min at 58°C; and primer extension: 2 min at 72°C) performed on a Pekin-Elmer-Cetus thermal cycler (Perkin-Elmer-Cetus, USA), 1 µl of the reaction mixture was transferred to the second reaction tube, containing the nested primer set under identical reaction conditions to those in the first reaction. After 40 cycles, 10 µl of the second reaction mixture was subjected to electrophoresis on 2% agarose gels (Seakem GTG, FMC Bioproducts, USA). Amplificates of both MIE-4 and pp150 target genes with the appropriate length were cloned in vector pUC19

digested with HindIII. One clone containing an amplicon as the insert was subjected to sequence analysis.

Sequence analysis

Initially, sequence analysis was undertaken with a single clone of each tissue sample, using the dideoxy-chain-termination kit as described by the manufacturer (Pharmacia, Uppsala, Sweden). five of the single clones that demonstrated sequence variation as compared to AD169 were analysed further. For this purpose, a 400 bp fragment of the MIE-4 gene and a 341-bp fragment of the pp150 gene were analyzed.

Fidelity considerations

To investigate the possibility of misincorporation of nucleotides during PCR, due to misreading by the polymerase, 18 separate tissue samples were spiked with purified DNA derived from human embryonic fibroblasts infected with the HCMV reference strain AD169. Nested PCR, cloning, and sequence analysis were carried out as for clinical samples with both nested primer sets.

Prevention of false-positive results

Pre- and post-PCR handling of samples and buffer preparation were done in different laboratories in order to prevent carry over of reaction products.

Results

Serology

All subjects had antibodies against HCMV, as detected by the latex agglutination assay and the ELISA. There was 100% agreement between both tests.

Nested PCR reaction

All tissue samples produced HCMV specific reaction products as demonstrated by agarose gel electrophoresis. Samples were positive when tested with MIE-4 or pp150 primers in separate assays, but also when tested in a duplex-PCR set-up. To test for carry over of reaction products, every second sample serving as a negative control containing only herring sperm DNA was also amplified, but none of these controls demonstrated PCR reactivity.

Fidelity considerations

Of the tissue samples spiked with HCMV reference strain AD169 infected fibroblast-derived DNA, a total of 16 independent amplicons of the MIE-4 gene were cloned and sequenced. Of these 16 cloned PCR products, 2 showed one mutation and 2 showed two mutations, all randomly distributed. Thus, 1 in 1066 nucleotides was misincorporated. Sixteen cloned PCR products of the pp150 gene were analyzed the same way. There were six mutations in 6 of the amplicons, giving a misreading rate of 1 in 909 nucleotides.

Sequence analysis

Most of the cloned PCR products derived from the tissue samples, had the same sequence as the HCMV reference strain AD169. However, 14/44 (table 2) did show some mutations (table 1). From these variant samples, but also from randomly chosen samples without mutations five additional clones were sequenced. Five additional clones of two tissue samples without mutations in the MIE-4 gene being the spleen of patient 1 and the liver of patient 6, remained without mutations as did additional samples without

mutations in the pp150 gene, being the spleen of patient 5 and the liver of patient 7. Only cloned PCR products obtained from the kidney of patient 5 consequently demonstrated mutations at positions 263, 332, 368, and 389 of the MIE-4 gene. Clones from other organs from the same patient did not show these variations. Within the pp150 gene, the same mutation at position 211 was observed in one clone from the kidney and in one clone from the liver from patient 1. The other mutations were located randomly throughout the MIE-4 and the pp150 amplicons. For the MIE-4 gene, fifty-six cloned PCR products were sequenced and 30 mutations (1 of 745 nucleotides) were observed. For the pp150 gene, seventy-six cloned PCR products were analysed, demonstrating a total of 20 (1 in 1292 nucleotides) mutations.

Table 1. Sequence analysis of six clones obtained from tissue samples showing sequence variation in the initial clone tested.

Tissue	MIE-4 mutations	PP150 mutations
Subject 1 spleen	none	5x AD169 . 321 (T)
kidney	3x AD169 . 135 (G) . 257 (T) . 306 (A)	5x AD169 . 211 (A) 235 (G)
liver	5x AD169 . 175 (T) 362 (G) 363 (G) 384 (A)	2x AD169 . 59 (G) 140 (C) . 227 (A) 106 (T) . 21 (G) 26 (C) 318 (A) 281 (A) 211 (A)
Subject 2 spleen	none	5x AD169 . 113 (A)
Subject 5 spleen	4x AD169 . 176 (G) . 212 (C)	none
kidney	. 263 (C) 332 (A) 368 (A) 389 (A) . 360 (G) 332 (A) 368 (A) 389 (A) . 94 (T) 130 (A) 141 (C) . 211 (G) 263 (C) 332 (A) 360 (G) 368 (A) 389 (A) . 170 (G) 180 (A) . 234 (C)	5x AD169
liver	5x AD169 . 275 (G)	4x AD169 . 157 (G) . 168 (A)
Subject 6 kidney	none	5x AD169 . 230 (T)
liver	none	5x AD169 . 68 (A)
Subject 7 spleen	none	5x AD169 . 16 (A) 21 (T)
kidney	none	

* none means that in the initial clone no variation in the sequences were detectable.

Discussion

In recent years it has been shown, that latent human cytomegalovirus (HCMV) is present in leucocytes (3, 4, 5, 6) and the arterial wall (7, 8, 9, 10) of healthy HCMV seropositive individuals. In this report we demonstrate by nested PCR, that HCMV is

also present in the kidney, spleen, and liver of previously healthy trauma victims. We also present sequence analysis data which demonstrate a high sequence homology between the HCMV present in these organs and the reference strain AD169, indicating that HCMV DNA and not DNA of another (Herpes)virus is detected.

The data presented in this study confirm the observation that HCMV DNA is present in a variety of organs obtained from HCMV seropositive individuals (20, 21, 22). Also in the mouse CMV model, a wide tissue distribution of latent MCMV has been demonstrated (24, 25).

Fidelity analysis of the procedures used demonstrated that 1 in 1066 nucleotides for MIE-4 and 1 in 909 nucleotides for pp150 were misincorporated when starting from AD169 sequences. This means that for MIE-4 a total of 21 mutations and for pp150 a total of 28 mutations could be expected on the basis on the estimated fidelity and the total number of fragments sequenced. The total number of mutations observed for MIE-4 and pp150 (30 and 20 respectively) is consistent with the estimated fidelity, so they probably do not represent true mutations. The random distribution of these mutations further adds to this conclusion. Other reports also demonstrate minor sequence variations within the MIE-4 gene (15), but provide no fidelity data. None of the observed mutations produces stop signals as analyzed by the PC gene program. The high degree of homology between the amplified fragments and the reference strain AD169 demonstrates that HCMV DNA is indeed detected, and the numerous negative controls exclude contamination with exogenous DNA.

The repeatedly observed mutations of the MIE-4 gene in the kidney of subject 5 and of the pp150 gene in the kidney and liver of subject 1 probably represent true mutations. Two types of the MIE-4 gene were present in the kidney of subject 5, and two types of the pp150 gene were detected in tissues from subject 1. Whether these two types of genes represent two different HCMV strains, however, is not known. Only isolation and propagation of HCMV from these organs can answer this question. During active infections, viral excretion of more than one strain has been demonstrated (23, 24, 25, 26), but this phenomenon has not been described during latency until now.

The presence of HCMV DNA organs that are used for transplantation and in the spleen suggests that the spleen can be used to screen for HCMV DNA by PCR, prior to transplantation. Seronegative organ recipients of organs containing two types of genes can then be carefully investigated for the emergence of HCMV strains carrying the same mutations.

References

1. Ho M. Cytomegalovirus biology and infection. New York; Plenum Medical Book Co., 1982.
2. Yow MD, Williamson DW, Leeds IJ, Thompson P, Woodward RM, Walms BF, Lester JW, Six HR, Griffiths PD. Epidemiological characteristics of cytomegalovirus infection in mothers and their infants. *Am J Obstet Gynecol* 1988;158:1189-1195.
3. Stanier P, Taylor DL, Kitchen AD, Wales N, Tryhorn Y, Tyms AS. Persistence of cytomegalovirus in mononuclear cells in peripheral blood from blood donors. *Br Med Journ* 1989;299:897-898.
4. Bevan IS, Daw RA, Day PJR, Ala FA, Walker MR. Polymerase chain reaction for detection of human cytomegalovirus infection in a blood donor population. *Br J Haematol* 1991;78:94-99.
5. Taylor-wiedeman J, Sissons JGP, Borysiewicz LK, Sinclair JH. Monocytes are a major site of persistence of human cytomegalovirus in peripheral blood mononuclear cells. *J Gen Virol* 1991;72: 2059-2064.

6. Taylor-Wiedeman J, Hayhurst GP, Siisons JGP, Sinclair JH. Polymorphonuclear cells are not sites of persistence of human cytomegalovirus in healthy individuals. *J Gen Virol* 1993;74:265-268.
7. Melnick JL, Petrie BL, Dreesman GR, Burek J, McCollum CH, Debakey ME. Cytomegalovirus antigen within human arterial smooth muscle cells. *The Lancet* 1983;ii:644-647.
8. Yamashiroya HM, Ghosh L, Yang R, Robertson L. Herpes viridae in the coronary arteries of young trauma victims. *Am J Pathol* 1988;130:71-79.
9. Hendrix MGR, Salimans MMM, van Boven CPA, Bruggeman CA. High prevalence of latently present cytomegalovirus in arterial walls of patients suffering from grade III atherosclerosis. *Am J Pathol* 1990;136:23-28.
10. Hendrix MGR, Daemen M, Bruggeman CA. Cytomegalovirus nucleic acid distribution within the human vascular tree. *Am J Pathol* 1991;138:563-567.
11. Bitch A, Kirchner H, Dupke R, Bein G. Failure to detect human cytomegalovirus DNA in peripheral blood leucocytes of healthy blood donors by the polymerase chain reaction. *Transfusion* 1992;32: 612-617.
12. Zhang LJ, Hauff P, Rutherford C, Churchill WH, Crumpacker CS. Detection of human cytomegalovirus DNA, RNA and antibody in normal donor blood. *J Infect Dis* 1995;171:1002-1006.
13. Chou S, Dennison KM. Analysis of interstrain variation in cytomegalovirus glycoprotein B sequences encoding neutralization related epitopes. *J I D* 1991;163:1-229-1234.
14. Fries BC, Chou S, Boeckh M, Torok-Storb B. Frequency distribution of cytomegalovirus envelope glycoprotein genotypes in bone marrow transplant recipients. *J Infect Dis* 1994;169:769-774.
15. Zaia JA, Gallez-Hawkins G, Churchill MA, Morton-Blackshere A, Pande H, Adler SP, Schmidt GM, Forman SJ. Comparative analysis of human cytomegalovirus alpha-sequences in multiple clinical isolates by using PCR and RFLP analysis. *J Clin Microbiol* 1990;28:2602-2607.
16. Chou S. Effect of interstrain variation on diagnostic DNA amplification of the cytomegalovirus major immediate early region. *J Clin Microbiol* 1992;30:2307-2310.
17. Verdonck LF, de Graan-Hentzen YCE, Dekker AW, Mudde GC, de Gast GC. Cytomegalovirus seronegative platelets and leucocyte poor red blood cells from random donors can prevent primary cytomegalovirus infection after bone marrow transplantation. *Bone Marrow Transplant* 1987;2:73-78.
18. Kraat YJ, Hendrix MGR, Landini MP, Bruggeman CA. Comparison of four techniques for detection of antibodies to CMV. *J Clin Microbiol* 1992;30:522-524.
19. Akrigg A, Wilkinson GWG, Oram JD. The structure of the major immediate early gene of human cytomegalovirus strain AD169. *Vir Res* 1986;2:107-121.
20. Jahn G, Kouzarides T, Mach M, Scholl BC, Plachter B, Traupe B, Freddie E, Satchwell SC, Fleckenstein B, Barrel BG. Map position and nucleotide sequence of the gene for the large structural phosphoprotein of human cytomegalovirus. *J virol* 1987;61:1358-136721.
21. Naoumov NV, O'Grady JG, Portman BC, Alexander GJM, Aldis P, Williams R. Rapid diagnosis of cytomegalovirus infection by in situ hybridization in liver grafts. *The Lancet* 1988;1(1899):1361-1364.
22. Arndt R, Dittmer R, Harfmann P, et al. Virus detection by DNA in situ hybridization in renal transplantation - a new perspective for differential diagnosis of virus infection versus rejection. *Transplant Proc* 1990; 22:1803-1804.
23. Myerson P, Hackman RC, Nelson JA, Ward DC, McDougal JK. Widespread presence of histologically occult cytomegalovirus. *Hum Pathol* 1984;15:430-439.
24. Klotman ME, Henry SC, Greene RC, Brazy PC, Klotman PE, Hamilton JD.

- Detection of mouse cytomegalovirus nucleic acid in latently infected mice by in vitro enzymatic amplification. *J Infect Dis* 1990;161:220-225.
25. Collins T, Pomeroy C, Jordan MC. Detection of latent cytomegalovirus DNA in diverse organs of mice. *J Infect Dis* 1993;168:725-729.
26. Chou S. Reactivation and recombination of multiple cytomegalovirus strains from individual organ donors. *J Infect Dis* 1989;160:11-15.
27. Shen C-Y, Chang S-F, Chao M-F, Huang E-S, Wu C-W. Identification of a serial change in recurrent cytomegalovirus strains in a healthy child by polymerase chain reaction. *J Infect Dis* 1993;168:252-253.
28. Spector SA, Hirata KK, Neuman TR. Identification of multiple cytomegalovirus strains in homosexual men with acquired immunodeficiency syndrome. *J Infect Dis* 1984;150:953-956.
29. Collier AC, Chandler SH, Handsfield HH, Corey L, McDougall JK. Identification of multiple strains of cytomegalovirus in homosexual men. *J Infect Dis* 1989; 159: 123-126.

CHAPTER 8

General discussion

1. Introduction
2. HCMV in atherosclerosis
3. Detection of HCMV in human tissues
4. Conclusions
5. References

1. Introduction

Human Cytomegalovirus (HCMV) a member of the Herpesvirusgroup (1) is ubiquitous in humans (2). It is generally accepted that after primary infection the HCMV remains present in the human body in a latent state (1, 2, 3, 4, 5). Although the concept of HCMV latency has been recognized some decades ago, little is known about the cell type or types that act as a reservoir for the latent virus. The possibility that the blood leucocyte contained latent HCMV was already considered in 1951 (6). HCMV was presumed to be present in these cells in an unidentified form from which it could only reactivate after allogeneic stimulus (7). Later on a definition of the latent state of HCMV was proposed by Banks and Rouse (8). They described it as a persistent infection in which the complete viral genome is present in a cell but no or very limited viral gene expression is found. Infectious viral particles are not produced. The capacity to reactivate from this latent state into an active infection was included in this definition by Fraser (9).

Since the introduction of molecular biological techniques, HCMV DNA and/or RNA was detectable in a variety of human tissues, like: Kaposi's sarcoma (10), carcinoma of the cervix (11, 12), prostate (13), and colon (14) angioimmunoblastic lymphadenopathy (15), and in atherosclerotic vascular walls (16, 17, 18, 19). The detection of HCMV nucleic acids in these laesions could suggest a role for this virus in the pathogenesis of these diseases, but it might just as well reflect a localization of latent HCMV. HCMV DNA could also be detected in leucocytes obtained from healthy blood donors (20, 21, 22) but data are conflicting since other investigators failed to detect HCMV DNA in these individuals (23, 24).

In this section we like to discuss our data concerning the presence of HCMV in atherosclerotic and non-atherosclerotic arterial walls and in various tissues obtained from healthy individuals, and compare our data with data from other groups.

2. HCMV in atherosclerosis

Studies with an avian herpesvirus, Marek's disease virus (MDV) in chickens demonstrated that an extensive atherosclerosis-like disease developed after infection with this virus (25). By in situ DNA hybridization, MDV nucleic acids could be detected in the vascular wall of infected chickens (25). Also in humans the presence of herpesviruses could be demonstrated in vascular walls obtained from patients undergoing reconstructive vascular surgery for occlusive atherosclerotic disease (15, 26). Both herpes simplex and HCMV were detected by in situ DNA hybridization (15, 26). Using an explant technique, HCMV antigens and DNA could be detected in smooth muscle cells cultured from atherosclerotic vascular tissues (16, 17) However no infectious virus could be recovered from these smooth muscle cell cultures. The detection of herpes viral nucleic acids including HCMV in the coronary artery and aorta of young trauma victims only demonstrating very early signs of atherosclerotic degeneration (19) suggested a role for these viruses in the development of atherosclerosis (27, 28). However, interpretation of these results in view of the pathogenesis of atherosclerosis is difficult.

A direct association between vasculopathy and HCMV comes from studies with human heart transplant recipients. In these studies a high rate of accelerated allograft arteriosclerosis was observed in HCMV seropositive recipients (29, 30, 31, 32). The development of a rat-model in which CMV infection doubles the rate of allograft atherosclerosis (33) opened the opportunity to study the effect of antiviral therapy on the development of allograft atherosclerosis. It was demonstrated that early treatment of CMV infected mice with DHPG (ganciclovir) could indeed prevent CMV infection-enhanced allograft

atherosclerosis (34, 35.) The observation that triple drug immunosuppression reduced CMV infection-enhanced allograft arteriosclerosis suggests that other factors than CMV infection play a role in the pathogenesis of this disease (36).

Also in immunocompetent patients restenosis of coronary arteries due to neointimal hyperplasia and arterial remodeling commonly occurs after coronary angioplasty and atherectomy (37, 38). In this group of patients, prior infection with HCMV has been demonstrated to be a strong independent risk factor for restenosis (39), suggesting a role for HCMV in the pathogenesis of this disease. A possible explanation for this phenomenon was found in the inhibition of the tumor suppressor protein p53 by a HCMV protein IE84 transcribed from the IE-1 and IE-2 genes (UL123) (40).

Some epidemiological data also suggest a role for HCMV in the pathogenesis of atherosclerotic disease of non-cardiac arteries. A high level of HCMV antibodies could be found in patients requiring vascular surgery (41). Also a graded relation between the levels of HCMV antibodies and the amount of intimal-medial thickening of the carotid artery could be demonstrated (42). However prior HCMV infection was not a risk factor for the development of primary coronary artery atherosclerosis (43).

Until now, no epidemiological studies have been performed using molecular techniques to describe the presence of HCMV DNA in arterial walls.

In our studies (chapter 3) we were able to demonstrate by PCR that 90% of the vascular samples obtained from patients undergoing reconstructive vascular surgery and representing end stage disease, contained HCMV DNA. However, 50% of the vascular samples obtained at autopsy from age and sex matched controls with no macroscopical signs of atherosclerosis did also contain HCMV DNA (44). In further studies (chapter 5) we found that HCMV DNA could be detected in all major elastic arteries, including pulmonary arteries which were microscopically free of atherosclerotic degeneration (45). Also in individual patients showing advanced atherosclerotic disease of at least 6 main vessels, arteries even not having intimal thickening were present and in all these arteries HCMV DNA could be detected (45). By using semi quantitative dot blot DNA hybridization (chapter 5) we were able to demonstrate that approximately 2 or less viral genomic equivalents (VGE) were present per 2500 human genomic equivalents (HGE) (45). There were no differences observed in the number of VGE per HGE present in arteries with or without atherosclerotic changes. Based on these observations we did not find a correlation between the presence of HCMV and atherosclerosis.

The presence of HCMV DNA in the vessel wall as described (chapters 3, 4, 5) is in accordance with most of the criteria for viral latency as defined by Banks and Rouse (8, 9) in which they describe viral latency as a persistent infection in which the complete viral genome is present but no or very limited viral gene expression is found; no infectious viral particles are produced, and the virus still must have the capacity to reactivate from this latent state into an active infection.

In our studies (chapter 2, 3, 4) we always used 2 different PCR targets to detect HCMV DNA in tissue samples. Since these PCR's were always simultaneously positive we concluded that the whole viral genome is present in the arterial samples. Using in situ hybridization with a probe reactive with immediate early sequences (UL122, UL123, UL125, UL126) (chapter 3, 4) we could demonstrate that a high number of arterial samples were positive. However, using a probe reactive with late (UL83) sequences only a limited number of samples showed a positive in situ hybridization (44, 46). These findings may suggest that HCMV transcription is limited to IE-genes and is not extended to late genes. Recently it has been demonstrated that also in bone marrow progenitor cells obtained from healthy seropositive donors the transcriptional program is also limited to IE-genes (34).

Despite numerous attempts we were not able to culture HCMV from the arterial samples. All these observations led us to the conclusion that the HCMV nucleic acids present in the human vascular wall represent latent virus. However we could not meet

the last criterium in the definition of HCMV latency since we could not demonstrate the capacity of this latent HCMV to reactivate into an active infection.

3. Detection of HCMV in human tissues

Our ability to detect nucleic acids of latent HCMV in arterial walls of healthy persons (chapter 3, 4, 5) suggested that in situ hybridization and PCR provide powerful tools for the detection of HCMV in other tissues. To study the presence of latent HCMV in other organs, only healthy subjects with no medical histories, like trauma victims which are available for donor nephrectomy, or individuals dying of sudden cardiac arrest were selected to provide tissue samples. To exclude the possibility of primary HCMV infection serum samples should be negative for HCMV-IgM antibodies. Until now only few reports meet these criteria. In a study from Toorkey et al (47) tissue sections obtained at autopsy from patients with trauma as the cause of death were investigated for the presence of HCMV. HCMV antigen was detected by immunohistochemical techniques using a monoclonal antibody reactive with a 68-kDa immediate early protein. HCMV antigens were detected in tubular and glomerular epithelial cells, cells of Bowmann's capsule, and endothelial cells of the kidney. Hepatocytes, kupffer cells, and endothelial cells of the liver contained HCMV antigens. In the lung, HCMV reactive cells consist of bronchial and alveolar epithelial cells, alveolar macrophages, fibroblasts and endothelial cells. In the spleen, T lymphocytes, macrophages, fibroblasts and endothelial cells were reactive, and finally in the brain, astrocytes and endothelial cells were HCMV positive. These results, however, could only be produced using a single batch of a monoclonal antibody reactive with a 72-kDa antigen of HCMV. By using another monoclonal antibody also reactive with a 72-kDa immediate early antigen of HCMV or a goat anti-serum raised against purified HCMV, they could not reproduce the results obtained with the first batch of antibody. The authors suggest that epitopes reactive with these last antibodies are destroyed by formalin. In our studies (chapter 5) HCMV was detected in tissues obtained from patients meeting the above criteria (48). HCMV was detected by in situ hybridization and PCR. Using in situ hybridization with a probe reactive with immediate early sequences of HCMV (UL122, UL123 UL125, UL126), we could demonstrate the presence of HCMV nucleic acids in various cell types. In the spleen, the red pulp was positive. In the kidney, HCMV positive cells were epithelial cells of the tubulus and cells of the glomerulus. Pancreatic cells showing hybridization were acinar cells and within the liver, HCMV containing cells were mainly hepatocytes. Our findings (48) are in agreement with the immunohistochemical finding of Toorkey et al (60) and demonstrate a wide tissue distribution of HCMV during latent infection. This distribution is similar to the tissue distribution as observed during active HCMV infection (49, 50, 51, 52, 53, 54).

We could also demonstrate the presence of HCMV DNA by PCR with 2 primer sets reactive with immediate early and late sequences of the virus (chapter 4, 5). Most reports using PCR for HCMV DNA detection confirm the identity of the PCR products by hybridization with a short, end-labelled oligonucleotide as probe. It therefore remains possible that significant sequence variation as described for the gB gene (55, 56), the LS-junction (57) but also the immediate early gene (UL123EX4) (58) which is currently in use as PCR target remains undetected. Sequence analysis can detect these variations but can also prove that indeed HCMV DNA is detected. In our studies (chapter 5) we did perform a sequence analysis of the amplicates produced by nested PCR of spleen, kidney and liver tissues obtained from trauma victims (59). Very few mutations were observed both in the immediate early (UL123) and in the PP150 (UL32) PCR targets as compared to the HCMV prototype strain AD169, demonstrating that indeed a patient-derived wild type HCMV is detected. The few mutations that were observed were randomly distributed over the amplified gene segment and did not seem

to produce stopcodons. The consistent finding of two genotypes for the immediate early gene in one individual and for the PP150 gene segment in another individual (59) possibly reflects latent infection with two HCMV strains. Also during active HCMV infection, viral excretion of more than one strain has been demonstrated (60, 61, 62, 63). Based on these observations we conclude that indeed HCMV DNA is detected and that during latency HCMV shows a wide tissue distribution. The HCMV tissue distribution is in sharp contrast with that of two other members of the herpes family: herpes simplex, which is only latently present in neurons of the spinal ganglia, and Epstein Barr virus latency which is only latently present in B-cells (1).

4. Conclusions

Latent HCMV shows a wide tissue distribution and is present in the splenic red pulp, in epithelial cells of the tubulus and cells of the glomerulus, in vascular smooth muscle cells, in pancreatic acinar cells and within the liver, HCMV-containing cells were mainly hepatocytes. This pattern closely resembles the wide distribution observed during active infection. Latent virus can be detected by in situ hybridization using probes reactive with immediate early sequences but not late sequences suggesting a very limited transcriptional program during latency.

The consistent finding that in a single individual, two HCMV genotypes are present suggests that during life not only infections with more than one HCMV strain are possible, but that these strains can also establish a latent infection within one host.

This widespread presence of HCMV DNA in normal tissues and also in atherosclerotic tissues cannot be interpreted in view of the pathogenesis of atherosclerosis since the presence of viral DNA possibly only reflects latent virus. However, the recent finding that also during latency HCMV IE sequences are transcribed and that IE-gene products can interfere with the tumor repressor protein p53 still opens intriguing possibilities for further studies onto the role of HCMV in the pathogenesis of diseases such as atherosclerosis, transplant arteriosclerosis and restenosis.

5. References

1. Fields BN, Knippe DM, Howley PM. Virology 2. Philadelphia; Lippincot Raven publishers 1996.
2. Ho M. Cytomegalovirus biology and infection. New York; Plenum Medical Book Co. 1991.
3. Jordan MC. Latent infection and the elusive cytomegalovirus. Rev.Infect.Dis. 1983;5:205-215.
4. Stevens JG. Human herpesviruses: a consideration of the latent state. Microbiol.Rev. 1989;53:318-332.
5. Bruggeman CA. Cytomegalovirus and latency: an overview. Vichows Archiv B.Cell.pathol. 1993;64:325-33.
6. Wyatt JP, Hemsath FA, Soash MD. Disseminated cytomegalic inclusion disease in an adult with primary refractory anaemia and transfusional sclerosis. Report of a case. Am.J.Clin.Path. 1951;21:50-55.
7. Jordan MC. Latent infection and the elusive cytomegalovirus. Rev.Infect.Dis. 1983;5:205-215.
8. Banks AB, Rouse BT. Herpesviruses - immune escape artists? Clin.Infect.Dis. 1992;14:933-941.
9. Fraser NW, Block TM, Spivack JG. The latency-associated transcripts of herpes simplex virus: RNA in research and function. Virology. 1992;191:1-8.
10. Boldogh I, Beth E, Huang ES, Kyalwazi SK, Giraldo G. Kaposi's sarcoma: detection of CMV DNA, CMV RNA, and CMNA in tumor biopsies. Int.J.Cancer 1981;28:-

469-474.

11. Huang E-S, Boldogh I, Mar EC. Evidence for possible association with human cancer. In: Phillips LA, ed. *Viruses associated with human cancer*. New York: Marcel Dekker 1983:161-194.
12. Huang E-S, Mar EC, Boldogh I, Baskar J. The oncogenicity of human cytomegalovirus. In Plotkin SA, Michelson S, Pagano JS, Rapp F, eds. *CMV: pathogenesis and prevention of human infections. Birth defects: original articles series, Vol. 20, no 1, March of Dimes Birth Defects Foundation*. New York: Alan R. Liss, 1984:193-211.
13. Geder L, Sanford EJ, Rohner TJ, Rapp F. Cytomegalovirus and cancer of the prostate: in vitro transformation of human cells. *Cancer Treat. Rep.* 1977;61:1-39146.
14. Huang E-S, Pagano JS. Cytomegalovirus DNA and adenocarcinoma of the colon: evidence for latent viral infection. *Lancet* 1978;i:957-960.
15. Yu A-M, Song R-L, Yu Z, Chen Y-P, Wang F-Z, Na L-X, Xie G-L, Yang M-J. detection of human cytomegalovirus antigen and DNA in lymph nodes and peripheral blood mononuclear cells of patients with angioimmunoblastic lymphadenopathy with dysproteinemia. *Arch. Pathol. Lab. Med.* 1992;116:490-494.
16. Melnick JL, Petrie BL, Dreesman GR, Burek J, McCollum CH, Debakey ME. Cytomegalovirus antigen within human arterial smooth muscle cells. *Lancet*;1983,ii:644-647.
17. Petrie BL, Melnick JL, Adam E, Burek J, McCollum CH, DeBakey ME. Nucleic acid sequences of cytomegalovirus in cells cultured from human arterial tissue. *J. Infect. Dis.* 1987;155:158-159.
18. Melnick JL, Hu C, Burek J, Adam E, Debakey ME. Cytomegalovirus DNA in arterial walls of patients with atherosclerosis. *J. Med. Virol.* 1994;42:170-174.
19. Yamashiroya HM, Ghosh L, Yang R, Robertson L. Herpes viridae in the coronary arteries of young trauma victims. *Am. J. Pathol.* 1988;130:71-79.
20. Stanier P, Taylor DL, Kitchen AD, Wales N, Tryhorn Y, Tyms AS. Persistence of cytomegalovirus in mononuclear cells in peripheral blood from blood donors. *Br. Med. J.* 1989;299:897-898.
21. Bevan IS, Daw RA, Day PJ, Ala FA, Walker MR. Polymerase chain reaction for the detection of human cytomegalovirus infection in a blood donor population. *Br. J. Haematol.* 1991;78:94-99.
22. Taylor-Wiedeman J, Sissons JGP, Borysiewicz LK, Sinclair JH. Monocytes are a major site of persistence of human cytomegalovirus in peripheral blood mononuclear cells. *J. Gen. Virol.* 1991;72:2059-2064.
23. Bitch A, Kirchner H, Dupke R, Bein G. Failure to detect human cytomegalovirus DNA in peripheral blood leucocytes of healthy blood donors by the polymerase chain reaction. *Transfusion* 1992;32:612-617.
24. Urushibara N, Kwon KW, Takahashi TA, Sekiguchi S. Human cytomegalovirus DNA is not detectable with nested double polymerase chain reaction in healthy blood donors. *Vox Sang* 1995;68:9-14.
25. Fabricant CG, Fabricant J, Milnick CR, Litrenta MM. Herpes virus-induced atherosclerosis in chickens. *Fed. Proc.* 1983;42:2476-2479.
26. Benditt EA, Barret T, McDougall JK. Viruses in the etiology of atherosclerosis. *Proc. Natl. Acad. Sci. USA* 1983;80:6386-6389.
27. Melnick JL, Adam E, DeBakey ME. Possible role of cytomegalovirus in atherogenesis. *J. A. M. A.* 1990;263:2204-2207.
28. Hajjar DP. Viral pathogenesis of atherosclerosis. Impact of molecular mimicry and viral genes. *Am. J. Path.* 1991;139:1195-1211.
29. Wreghit TG, Hakim M, Gray JJ, Kucia S, Cory-Pearce R, Wallwork J, English TAH. A detailed study of cytomegalovirus infections in the first 160 heart/lung

- transplant recipients at Papworth Hospital, Cambridge England. *Transplant. Proc.* 1987;19:2495-2496.
30. Grattan MT, Moreno-Gabral CE, Starnes VE, Over PE, Stinson EB, Shumway NE. Cytomegalovirus infection is associated with cardiac allograft rejection and atherosclerosis. *JAMA* 1989;261:3561-3566.
 31. McDonald K, Rector TS, Braunlin TA, Kubo SH, Olvari MT. Association of coronary artery disease in cardiac transplant recipients with cytomegalovirus infection. *Am.J.Pathol.* 1989;64:359-362.
 32. Zhou YF, Leon MB, Waclawiw MA, Popma JJ, Yu ZX, Finkel T, Epstein SE. Association between prior cytomegalovirus infection and the risk of restenosis after coronary atherectomy. *N. Engl. J. Med.* 1996;29:624-768.
 33. Lemstrom KB, Bruning JH, Bruggeman CA, Lautenschlager IT, Hayry PJ. Cytomegalovirus infection enhances smooth muscle cell proliferation and intimal thickening of rat aortic allografts. *J.Clin.Invest.* 1993;92:549-558.
 34. Lemstrom KB, Bruning JH, Bruggeman CA, Koskinen PK, Aho PT, Yilmaz S, Lautenschlager IT, Hayry PJ. Cytomegalovirus infection-enhanced allograft arteriosclerosis is prevented by DHPG prophylaxis in the rat. *Circulation* 1994;90:1969-1978.
 35. Lemstrom K, Sihvola R, Bruggeman CA, Hayry P, Koskinen P. Cytomegalovirus infection-enhanced cardiac allograft vasculopathy is abolished by DHPG prophylaxis in the rat. *Circulation* 1997;95:1614-2616.
 36. Lemstrom KB, Bruning JH, Bruggeman CA, Lautenschlager IT, Hayry PJ. Triple drug immunosuppression significantly reduces immune activation and allograft arteriosclerosis in cytomegalovirus-infected rat aortic allografts and induces early latency of viral infection. *Am.J.Pathol.* 1994;144:1334-1347.
 37. Serruys PW, Luyten HE, Beatt KJ. Incidence of restenosis after succesful coronary angioplasty: a time related phenomenon : a quantitative angiographic study in 342 consecutive patients at 1, 2, 3, and 4 month. *Circulation* 1988;77:-361-71.
 38. Faxon DP, Currier JW. prevention of post-PTCA restenosis. *Ann.N.Y.Acad.Sci.* 1995;748:419-27.
 39. Zhou YF, Leon MB, Waclawiw MA, Popma JJ, Yu ZX, Finkel T, Epstein SE. Association between prior cytomegalovirus infection and the risk of restenosis aftet coronary atherectomy.N.Eng.J.Med. 1996;335:624-630.
 40. Speir E, Modali R, Huang ES, Leon MB, Shawl F, Finkel T, Epstein SE. Potential role of human cytomegalovirus and p53 interaction in coronary restenosis. *Science* 1994;265:391-394.
 41. Adam E, Melnick JL, Probstfield JL, Petrie BL, Burek J, Bailey KR, McCollum CH, DeBakey ME. High level of cytomegalovirus antibody in patients requiring vascular surgery for atherosclerosis. *Lancet* 1987;2:291-293.
 42. Nieto FJN, Adam E, Sorlie P, Farzadegan H, Melnick JL, Comstock GW, Szklo M. Cohort study cytomegalovirus infection as a risk factor for carotid intimal-medial thickening, a measure of subclinical atherosclerosis. *Circulation* 1996;94:-922-927
 43. Adler SP, Hur JK, Wang JB, Vetrovec GW. Prior infection with cytomegalovirus is not a major risk factor for angiographically demonstrated coronary artery atherosclerosis. *J.Infect.Dis* 1998;177:209-212.
 44. Hendrix MGR, Salimans MMM, van Boven CPA, Bruggeman CA. High prevalence of latently present cytomegalovirus in arterial walls of patients suffering from grade III atherosclerosis. *Am.J.Pathol.* 1990;136:23-18.
 45. Hendrix MGR, Daemen M, Bruggeman CA. Cytomegalovirus nucleic acid distribution within the human vascular tree. *Am.J.Pathol.* 1991;183:563-567.
 46. Hendrix MGR, Dorman PHJ, Kitslaar P, Bosman F, Bruggeman CA. The presen-

- ce of CMV nucleic acids on arterial walls of atherosclerotic and non-atherosclerotic patients. *Am.J.Pathol.* 1989;134:1151-1157.
47. Toorkey CB, Carrigan DR. Immunohistochemical detection of an immediate early antigen of human cytomegalovirus in normal tissues. *J.Infect.Dis.* 1989;160:741-751.
 48. Hendrix MGR, Wagenaar M, Slobbe R, Bruggeman CA. Widespread presence of cytomegalovirus DNA in tissues of previously healthy trauma victims. *J. Clin. Pathol.* 1997;50:59-63.
 49. Andersen CB. Detection of cytomegalovirus-infected cells in autopsy material by in situ hybridization. *A.P.M.I.S.* 1990;98:363-368.
 50. Gnann JW, Ahlmen J, Svalander C, Olding L, Oldstone MBA, Nelson JA. Inflammatory cells in transplanted kidneys are infected by human cytomegalovirus. *A.J.P* 1988;132:239-248.
 51. Borisch B, Jahn G, Scholl BC, Filger-Brillinger J, Heymer B, Fleckenstein B, Muller-Hermelink HK. Detection of human cytomegalovirus DNA and viral antigens in tissues of different manifestations of CMV infection. *Virch.Arch.B.Cell.Pathol.* 1988;55:93-99.
 52. Wolber RA, Lloyd RV. Cytomegalovirus detection by nonisotopic in situ DNA hybridization and viral antigen immunostaining using a two-color technique. *Hum.Path.* 1988;19:736-741.
 53. Meyerson D, Hackman RC, Nelson JA, Ward DC, McDougall JK. Widespread presence of histologically occult cytomegalovirus. *Hum.Path.* 1984;15:430-439.
 54. Weiss LM, Movahed LA, Berry GJ, Billingham ME. In situ hybridization studies for viral nucleic acids in heart and lung allograft biopsies. *A.J.P.* 1990;93:675-679.
 55. Chou S, Dennison KM. Analysis of interstrain variation in cytomegalovirus glycoprotein B sequences encoding neutralization related epitopes. *J I D* 1991;163:1-229-1234.
 56. Fries BC, Chou S, Boeckh M, Torok-Storb B. Frequency distribution of cytomegalovirus envelope glycoprotein genotypes in bone marrow transplant recipients. *J Infect Dis* 1994;169:769-774.
 57. Zaia JA, Gallez-Hawkins G, Churchill MA, Morton-Blackshere A, Pande H, Adler SP, Schmidt GM, Forman SJ. Comparative analysis of human cytomegalovirus alpha-sequences in multiple clinical isolates by using PCR and RFLP analysis. *J Clin Microbiol* 1990;28:2602-2607.
 58. Chou S. Effect of interstrain variation on diagnostic DNA amplification of the cytomegalovirus major immediate early region. *J Clin Microbiol* 1992;30:2307-2310.
 59. Hendrix MGR, Beuken E, Slobbe RL, Bruggeman CA. Detection and sequence analysis of the major immediate early and PP150 gene of latent human cytomegalovirus in spleen, liver and kidney tissues of trauma victims. *J. Med. Virol.* 1996;50:193-197.
 60. Chou S. Reactivation and recombination of multiple cytomegalovirus strains from individual organ donors. *J Infect Dis* 1989; 160: 11-15.
 61. Shen C-Y, Chang S-F, Chao M-F, Huang E-S, Wu C-W. Identification of a serial change in recurrent cytomegalovirus strains in a healthy child by polymerase chain reaction. *J Infect Dis* 1993; 168: 252-253.
 62. Spector SA, Hirata KK, Neuman TR. Identification of multiple cytomegalovirus strains in homosexual men with acquired immunodeficiency syndrome. *J Infect Dis* 1984; 150: 953-956.
 63. Collier AC, Chandler SH, Handsfield HH, Corey L, McDougall JK. Identification of multiple strains of cytomegalovirus in homosexual men. *J Infect Dis* 1989; 159: 123-126.

Samenvatting

Infecties met cytomegalovirus (CMV), een virus dat tot de familie van de herpesvirussen behoort, komen bij de mens vaak voor. Zo heeft 40 tot 90% van de volwassenen aantoonbare antistoffen tegen dit virus. Meestal verlopen deze infecties zonder merkbare gevolgen maar in sommige gevallen kan er een mononucleosis-achtig beeld ontstaan. Na de primaire infectie blijft het virus levenslang in een sluimerende toestand binnen een gastheer aanwezig. Tijdens deze sluimerende toestand, latente infectie genaamd, vindt er geen virusreproductie plaats en wordt hooguit een zeer klein deel van het virale genoom afgelezen. Waar, dat wil zeggen in welke cellen, het virus zich tijdens deze latente infectie bevindt, is nog steeds onduidelijk. Reactivatie vanuit deze latente toestand, waarbij weer actieve virusvermeerdering plaatsvindt, treedt vooral op bij personen met een gestoorde immuniteit, hetzij door de weerstandverlagende behandeling na orgaantransplantatie, of als gevolg van AIDS. CMV-reactivaties binnen deze patientengroepen veroorzaken vaak ernstige, en soms dodelijke, ziekteverschijnselen.

De gedachte dat er naast de bekende door CMV veroorzaakte ziektebeelden ook een verband tussen CMV en atherosclerose zou kunnen zijn, is gebaseerd op een aantal observaties. Zo veroorzaakt Marek's disease virus (MDV), een op CMV lijkend herpesvirus van vogels, bij kippen atherosclerose van de grote vaten. Met behulp van in situ DNA-hybridisatie is het mogelijk MDV-nucleinezuren in de atherosclerotische kippenvaatwand aan te tonen. Spoedig na deze ontdekking werd met in situ DNA-hybridisatie CMV aangetoond in vaatwanden van patienten die een reconstructie van de grote vaten wegens ernstige atherosclerose ondergingen. CMV kon echter ook worden aangetoond in de coronairvaten van jonge patienten zonder atherosclerotische degeneratie. Of CMV een rol speelt in de pathogenese van atherosclerose kan op basis van deze observaties niet worden geconcludeerd. Het is namelijk ook mogelijk dat de gladde spiercellen in de humane vaatwand een reservoir voor latent CMV zijn. Meer directe aanwijzingen dat CMV een rol zou kunnen spelen bij atherosclerose komen van andere observaties. Zo zal na een harttransplantatie een deel van de patienten een versnelde vorm van atherosclerose van de coronairvaten van het getransplanteerde hart doormaken. Deze versnelde vorm van atherosclerose treedt hoofdzakelijk op bij patienten die CMV-seropositief zijn. Ook is er inmiddels een rattenmodel ontwikkeld waar atherosclerose ontstaat na een infectie met een ratten-cytomegalovirus. Binnen dit model bleek het mogelijk om met behulp van antivirale therapie het ontstaan van atherosclerose na CMV infectie volledig te voorkomen.

Om nu te onderzoeken of er een verband bestaat tussen CMV en atherosclerose, of dat de humane vaatwand een reservoir voor virale latentie is, hebben we vaatwandbiopten bij twee verschillende patientengroepen genomen, en deze met moleculaire technieken, op de aanwezigheid van CMV-nucleinezuren onderzocht. De onderzoeksgroep bestond uit patienten die vanwege ernstige atherosclerotische afwijkingen (graad III) een electieve reconstructie van een groot vat ondergingen. Hierbij werd meestal een biopt van de Aorta abdominalis en soms van de Arteria femoralis genomen. Een controle-groep bestond uit gematchte patienten bij wie tijdens obductie was vastgesteld dat er vrijwel geen atherosclerose (maximaal graad I) aanwezig was en bij wie een biopt van de Aorta abdominalis werd genomen. Met behulp van de polymerase kettingreactie (PCR) kon in 90% van de atherosclerotische vaatwanden CMV worden aangetoond, versus in 50% van de controle vaatwanden. De plaats waar de CMV-nucleinezuren zich bevonden, aangetoond met in situ DNA-hybridisatie, bleek in beide patientengroepen echter niet de degeneratieve vaatwand te zijn, maar juist dat deel van de vaatwand dat histologisch normaal was. Het CMV bleek in de gladde spiercellen aanwezig te zijn. Om te bekijken of CMV ook in andere arterien aanwezig is, werd een derde patientengroep verzameld waarbij tijdens obductie een biopt van alle elastische arterien werd genomen. Analyse van deze biopten toonde aan dat CMV in de vaatwand van alle grote vaten

aanwezig is, en tevens dat er geen enkele relatie met atherosclerotische verandering is. De hoeveelheid virus in al deze vaten was onafhankelijk van de mate van atherosclerose en was minder dan 2 virusdeeltjes per 2500 cellen. Op basis van onze gegevens is het dus niet mogelijk een relatie tussen CMV en atherosclerose aan te tonen. De aanwezigheid van CMV in alle vaatwanden betekent mogelijk wel dat de lang gezochte plaats voor dit virus tijdens zijn latente fase gevonden kan zijn.

Tijdens deze latente fase vindt er geen virusreproductie plaats en wordt hooguit een zeer klein deel van het virale genoom afgelezen. Dat er in de vaatwanden geen virusreproductie plaatsvindt hebben we kunnen afleiden uit het feit dat we ondanks tientallen pogingen geen prolifererend CMV hebben kunnen isoleren. Wel hebben we aannemelijk kunnen maken, zowel met de PCR als met dot blot DNA-hybridisatie, dat waarschijnlijk het hele virale genoom in de vaatwand aanwezig is, en dus dat de mogelijkheid tot reactivatie in ieder geval genetisch bestaat. Dat er maar een klein deel van het genoom wordt afgelezen hebben we aangetoond met in situ DNA-hybridisatie. Alleen mRNA afgelezen van immediate early regio's van het genoom kon worden aangetoond terwijl mRNA afgelezen van andere regio's die met name tijdens de virusreproductie actief zijn, niet kon worden aangetoond. Op basis van deze gegevens concluderen we dat de humane vaatwand een reservoir voor latent CMV is.

Omdat tijdens een acute CMV-infectie het virus in vrijwel alle organen aanwezig is dringt zich al snel de vraag op of het virus tijdens de latente fase ook in alle organen aantoonbaar is. Zowel met de PCR als met in situ DNA-hybridisatie was het inderdaad mogelijk CMV aan te tonen in een groot aantal organen en celtypes. Zo was het virus aantoonbaar in de gladde spiercellen van de vaatwand, hepatocyten, tubulaire en glomerulaire niercellen, cellen in de rode pulpa van de milt en in acinaire cellen van het pancreas. Om actieve virusproliferatie uit te sluiten zijn al deze biopten immunohistochemisch onderzocht op de aanwezigheid van virale eiwitten met behulp van monoclonale antilichamen, die gericht zijn tegen specifieke CMV-eiwitten. Deze werden echter nooit aangetoond. Op basis van deze gegevens concluderen we dat latent CMV in vele organen en celtypes aanwezig is.

Humane herpesvirussen vertonen veel overeenkomsten op DNA-niveau. Om aan te tonen dat HCMV en niet een ander herpesvirus in deze organen aanwezig was, werd het noodzakelijk de DNA-fragmenten die met de PCR uit de diverse organen geamplificeerd werden verder te analyseren. We hebben ervoor gekozen een sequentie-analyse van een groot deel van deze amplificaten uit te voeren. Het resultaat was dat er meer dan 95% homologie was met een HCMV prototypestam, aantonend dat inderdaad HCMV in de biopten aanwezig was. In een enkele patient werden consequent twee varianten van het virus aangetroffen, hetgeen suggereert dat een patient latent geïnfecteerd kan zijn met meer dan één stam.

In conclusie:

Hoewel op basis van andere studies een rol voor CMV in de pathogenese van atherosclerose tot de mogelijkheden behoort, hebben wij in ons systeem geen directe relatie tussen HCMV en atherosclerose kunnen aantonen. Wel hebben we aannemelijk kunnen maken dat in de latente fase HCMV in vele organen en celtypes, waaronder gladde spiercellen in de vaatwand, aanwezig is.

Curriculum Vitae

Matheus Gerardus Ronald (Ron) Hendrix

28 november 1958 geboren te Maastricht

1978	Eindexamen Atheneum B, St. Maartenscollege te Maastricht
1979-1980	Vervulling militaire dienstplicht
1980-1984	Doctoraalstudie geneeskunde
1986	Doctoraal diploma geneeskunde (6 oktober)
1987	artsexamen, Rijksuniversiteit Limburg te Maastricht
1987-1991	Assistent in opleiding (AIO), medische microbiologie, Rijksuniversiteit Limburg te Maastricht o.l.v. Mw. Prof. Dr. C.A. Bruggeman
1991-1994	Opleiding medische microbiologie aan het Academisch Ziekenhuis Maastricht. Opleiders: Prof. Dr. C.P.A. van Boven en Dr. F. van Tiel
1994	Inschrijving specialisten register
1994	Arts-microbioloog Stichting Streeklaboratoria voor de volksgezondheid in Twente en de Gelderse Achterhoek te Enschede
1997	Benoeming tot medisch directeur/hoofd laboratorium Stichting Streeklaboratoria voor de volksgezondheid in Twente en de Gelderse Achterhoek te Enschede

Nawoord

Het hier beschreven onderzoek is hoofdzakelijk uitgevoerd binnen de vakgroep medische microbiologie. Echter, ook de vakgroepen heelkunde, pathologie en celbiologie van de universiteit Maastricht en tevens de pathologen van het De Wever ziekenhuis te Heerlen, de pathologen van Het Maaslandziekenhuis te Sittard en de stafleden van het centraal klinisch virologisch laboratorium van het Academisch Ziekenhuis Leiden hebben een wezenlijke bijdrage aan het tot stand komen van dit proefschrift geleverd.

Binnen deze grote groep mensen is er een aantal die ik apart wil bedanken voor de belangrijke rol die ze hebben gespeeld.

Allereerst natuurlijk mijn promotor Cathrien Bruggeman. Beste Cathrien, ik bedank je natuurlijk voor de begeleiding tijdens mijn promotieonderzoek, maar vooral ook voor de periode ervoor (macrofagen) en erna (het maken van het boekje). Wat ik vooral waardeer is de grote mate van vrijheid die ik tijdens mijn onderzoek heb gekregen. Gedurende de lange tijd dat we elkaar inmiddels kennen heb ik in velerlei opzicht van je geleerd.

Prof. Dr. C.P.A. van Boven. Geachte Cees, zoals altijd op de achtergrond aanwezig maar met grote invloed. Mijn liefde voor de microbiologie heb ik ongetwijfeld van jou meegekregen.

Peter Dormans, dank je voor de (forse) hulp die jij me gaf tijdens mijn eerste stappen op het terrein van de praktische moleculaire microbiologie. Zonder deze hulp zou dit deel van de microbiologie altijd boekenkennis zijn gebleven.

Prof. Dr. P.J.E.H.M. Kitslaar, wiens enthousiasme ervoor zorgde dat alle vaatoperaties onder mijn aandacht werden gebracht, zodat de patiëntengroepen konden worden samengesteld.

Marcel Salimans die ervoor zorgde dat ik vanaf het prille begin betrokken ben geraakt bij de PCR. Marcel, niet alleen jouw vakkennis maar ook je enthousiasme zijn zeer waardevol gebleken. Jouw bemoeienissen hadden zelfs een gigantische invloed op mijn privésituatie.

Math Daemen, jouw snelle bereidheid om in het onderzoek te participeren, maar ook de talrijke vruchtbare discussies zijn van groot belang gebleken.

Mariëtte Wagenaar, jouw constante doorvragen heeft me menigmaal tot herbezinning gedwongen en jouw inzet en enthousiasme waren verfrissend.

Rob Slobbe, ondanks het feit dat je in een relatief laat stadium bij mijn promotieonderzoek betrokken bent geraakt, kan ik alleen maar met genoegen aan jouw inbreng terugdenken. Niet alleen kon je een nieuwe wending in het onderzoek aanbrengen, maar kon je tevens veel moleculaire kennis overdragen.

Eric Beuken, jouw rustige en bekwame manier van werken kwam precies op een goed moment tijdens de afsluiting van de onderzoeksfase.

And last, but certainly not least, Wil Mullers. Wat je bijgedragen hebt kan ik niet precies benoemen, maar mijn vaste overtuiging is dat zonder jouw inbreng, je relativerende

levenshouding en je inzet heel veel op heel veel gebieden niet goed was gegaan.

Naast dit persoonlijke dankwoord is het op zijn plaats de hele gemeenschap die een vakgroep toch eigenlijk is te bedanken voor hun bijdrage. Zonder mensen als Gert Grauls, Fia Claus, Yvette Kraat, Frans Stals, Maaïke Persoons, Jan Jacobs, Frank van Tiel en Ellen Stobbering zou er geen onderzoeksklimaat zijn.

Ook wil ik mijn huidige collega's in Enschede en met name Wim Severin bedanken voor de tijd die zij mij gunden om in alle rust een aantal artikelen en mijn proefschrift af te kunnen schrijven.

En natuurlijk Esther....