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DETECTION OF CYTOMEGALOVIRUS IN HUMAN CELLS

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ABSTRACT

lnfection of the host by cytomegaloviruses (CMVs) can Iead to productive infection that can either persist asymptomatically, be accompanied by disease, or become latent. Although there are a number of excellent current reviews on one or another aspect of cytomegalovirus, the last comprehensive treatment of this subject was that of Krech et al. (197la). In view of the amazing advances in the virological, epidemiologic, and clinical knowledge of cytomegaloviruses, an up-to-date articl is needed.

Such a work should cover many areas of expertise and a voluminous technical literature. Each area might have been reviewed and analyzed by workers more expert than myself. I have tried to review the Iiterature and provide a critical summary for each area discussed. To do this, I provide as much of the primary data of the relevant works as needed and not just the qualitative conclusions. Inevitably, the flow of the narrative may be interrupted by dry facts and figures.

However, such information is essential to make this a meaningful reference work. But for those not interested in such details, I have provided at what I hope are crucial points critiques and summaries.

This book is not an exhaustive review of all the literature. This is probably no Ionger possible or even desirable. By selection, however, one runs the risk of having missed or ignored important papers. I am keenly aware of this, and I wish to apologize for such oversight, if that is possible. I visualize this work as being useful for physicians, scientists, and students interested in the biology of and infection by human cytomegalovirus.

I feel that to understand these, the basic virology of this virus and related herpesviruses must be included. Topics that have not advanced a great deal and that are adequately described in available works, such as the anatomic pathology, have not been reviewed in detail. The emphasis has been placed on newer insights in the virology, immunology, serology, epidemiology, and especially clinical aspects of human cytomegalovirus.

Keywords: cytomegalovirus, Detection, immunology, serology

INTRODUCTION

In 1904, Ribbert (1904) wrote that in 1881 he saw 1arge "protozoan like" cells in the sections of a kidney of an alleged leutic stillbom. He was unable to interpret bis observation until he saw the report of Jesionek and Kiolemenoglou (1904), who bad noted for the firsttime the presence of protozoanlike cells in the lungs, kidneys, and liver of an 8-month leutic fetus (Fig. 1.1).

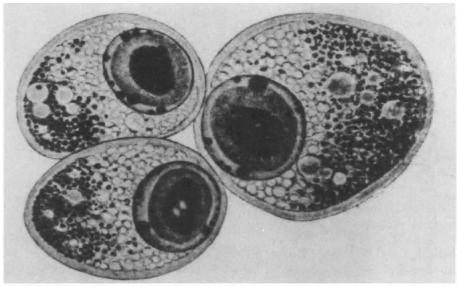

Figure 1.1. "Protozoan" -like cells seen in the kidney, lung, and Ii ver of an 8-month-old 1eutic fetus by Jesionek and Kio1emenglou (1904). (By permission of Münchener Medizinische Wochenschrift.)

The experimental relationship between herpes encephalitis infection in the rabbit and intranuclear inclusions was demonstrated by Goodpasture (1925). Although the protozoa theory was kept alive in Germany until 1930 (Wagner, 1930), the viral theory based on Lipscheutz' concepts gradually prevailed. Acceptance was accelerated by the work of Cole and Kuttner (1926) on the transmissibility of guinea pig salivary gland virus (see chapter 15) and the experimental and pathological works of Cowdry (1934), who definitively described the relationship between inclusion bodies and certain virus infections (1).

The isolation of human CMV was not possible until human cells could be routinely grown in cultures (Enders et al., 1949), since the cytomegaloviruses are largely species specific, and human CMV does not grow in experimental animals (2). When such culture techniques became commonplace in the early 1950s, it became a question of time until isolation was achieved (3).

Cytomegalovirus infection is diagnosed by isolation of the virus or by demonstrating its presence by serologic or molecular techniques. Serologic diagnosis (see Chapter 6) is accurate and specific but may be of limited usefulness in acute medicine because the required enhances in antibody state or titers may take too long to demonstrate (4).

MATERIALS AND METHODS

Virological Diagnosis in the Presence of Antibody

One of the hallmarks of CMV infection is persistence of the virus in various body fluids despite the development of serum neutralizing antibodies (see Sections 11.2 and 12.3). One possible reason is the protective role ß2 -microglobulin might play against serum neutralization (McKeating et al., 1987) (Section 4.5.2) (Fig 4). In CMV mononucleosis, whether arising spontaneously or after transfusion, and particularly in CMV infection in the immune-suppressed patient, viremia may persist for months despite the presence of serum antibodies (Stulberg et al., 1966; Lang and Hanshaw, 1969). There have been sturlies on the coappearance of CMV and antibodies in other body fluids. Cytomegalovirus viremia is common in patients with AIDS, who are almost invariably seropositive for CMV. Tamura et al. (l980a) studies saliva for the presence of neutralizing antibodies against hCMV (5). A microculture plaque assay without complement was performed. Out of 54 infants from 3 months to 3 years of age who were viruric or seropositive, 41 (75%) had neutralizing antibodies in their saliva despite presence of CMV in the saliva. No antibodies to virus were found in the saliva of 11 seronegative controls. The neutralizing titer of saliva was 1 : 4 to 1 : 32, whereas corresponding serum neutralizing titers were 1 : 32 to I : 512. The amount of antibody in saliva was apparently inadequate to neutralize the virus. Waner et al. (1977) studied I 02 specimens of cervical secretions for lgG and lgA antibody to CMV detected by immunofluorescence. Specimens from six of ten patients who were excreting CMV contained IgG antibody. Four of the six samples also contained lgA antibodies against CMV(6). Only three samples positive

for lgG antibodies were from patients who were not virus excreters, although it is possible that viral excretion might have been detected if more samples were cultured from these patients. As in saliva, the presence of antibody in cervical secretion may be associated with the presence of virus (Fig 1).

Polymerase Chain Reaction

The polymerase chain reaction (PCR) amplifies minuscule amounts of DNA in a clinical specimen and makes them detectable. lt takes advantage of a heat-stable DNA polymerase, which assembles a complementary strand from a defined segment in a strand of DNA, a primer. In order to do this, PCR requires first that a double-stranded DNA be denatured. Then the primer segment is added, annealed to a denatured DNA strand, and extended in the presence of DNA polymerase (7). Saiki et al. (1988) described a system for amplifying and concentrating specific sequences as much as 106-fold. lt has become a powerful tool and has facilitated the detection, characterization, and sequencing of increasing numbers of viruses, including human papilloma virus, HIV, HTLV-1, HTLV-11, and lentiviruses (Schochetman et al., 1988). Shibata et al. (1988) detected CMV DNA in the blood of 14 of 27 patients with HIV infection or AIDS. It was more sensitive than standard culture assays and used only 20 J.Ll of blood. Interestingly, no CMV DNA was detected in the blood of healthy seropositive individuals. This is surprising in view of the reports that mononuclear cells of normal seropositive subjects contain CMV transcripts (Schrier et al., 1985; see Section 5.5.3) (8). Demmler et al. (1988) reported results of testing urine samples from 44 congenitally infected infants. Synthetic oligonucleotide primer pairs were used to amplify DNA from the immediate early and late antigen regions of the CMV genome. Amplified products were detected by dot-blot hybridization. When compared with tissue culture, sensitivity and specificity were 100%. Detection by gel electrophoresis provided a sensitivity of93%. Both IEA and LA primers were needed to detect virus in all 46 samples (Fig 2).

DEMONSTRATION OF CMV BY OTHER METHODS

For rapid diagnosis, a drop of urine or urinary sediment may be smeared on a slide and fixed for 20 min with equal parts of ether and alcohol. After being stained with hematoxylin and eosin, Giemsa, or Papanicolaou stains, the slide is examined for inclusion-containing cells (Fetterman, 1952) (Fig. 5.4) (9). This simple early procedure may be used in suspected cases of congenital infection or cytomegalic inclusion disease,

but it is not now in general use. It Iacks both sensitivity and specificity (10). Electron microscopy has been used as a rapid and reliable method of diagnosis for certain types of virus infections, notably, smallpox, rotaviruses, Norwalk agents of diarrhea, and hepatitis A and B viruses (Schmidt and Emmons, 1989). The feasibility of diagnosing CMV infection in infants by electron microscopy was studied by Lee et al. (1978). Examination for CMV in urine after preparation of the specimen by the "pseudoreplica" technique was completed in 15 to 30 min. This technique permits tenfold concentration ofthe specimen. When the virus concentration in the urine was greater than 104 PFU/ml, this method was consistently reliable. However, if the virus concentration was lower, or if a distinction from other herpesviruses (such as herpes simplex virus) was required, then it was less satisfactory. The specificity of this method may be improved by immune electron microscopy. These methods are not in general use (11).

RESULTS

Hsia *et al*. (1989) selected for amplification fragments of 130 and 152 base pairs (bp) at two opposite ends of Eco RI fragment D of the AD169 strain of CMV. This fragment is transcribed early and also late in abundance. After amplification of the 152bp DNA, only one CMV-infected fibroblast cell or 0.01 pg of fragment D DNA was detected (12). All 35 culture-positive urine samples were detected, and two culture-negative samples were positive only by PCR. Urine samples from these two patients also became positive later, suggesting that PCR was more sensitive than routine cultural methods. The full potential of PCR has not yet been realized. One of the major problems in the CMV field is the difficulty of predicting transmissibility of CMV from the blood or tissues of seropositive subjects. Blood from seropositive donors does not always transmit CMV (see Sections 9. 7.1 and 13 .1.1). A similar problern exists in the case of transmission of CMV by donor organs or tissues in transplantation (Section 13.1.1). lt is possible that an appropriate type of quantitative PCR or reverse transcriptase PCR in which RNA transcripts rather than DNA are amplified may be helpful. 1t is also possible that in detecting blood, blood products, or other tissues that might harbor transmissible CMV, seropositivity of the donor may also miss certain cases of occult infection. Stanier et al. (1989) reported that five blood samples from 25 PCR-positive specimens came from seronegative individuals (Teble 1).

Antigenemia has been tested for its ability to be a surrogate for positive blood cultures. Van der Bij et al. (1988) used a mixture of different monoclonal antibodies to detect antigenemia in patients with viremia . Inclusion of antibodies against IEA was found tobe essential for sensitivity. They assessed 139 blood samples from 15 transplant patients. Cytomegalovirus viremia was documented in 23 samples, and antigenemia was found in 44 samples. All positive samples were from nine patients diagnosedas having active CMV infection. Twenty-one of 23 viremic samples were also antigenemic. Of the remaining 23 antigenemic samples, 20 came from the previremic phase of acute disease, and three came from two patients with serologic evidence of infection (13,14). These results suggest that direct detection of CMV antigens in a cytospin preparation of peripheral leukocytes is specific and possibly more sensitive than routine blood cultures, particularly before the onset of viremia. The procedure is also rapid and potentially more cost effective . Positive cells were mostly polymorphonuclear cells with nuclear or perinuclear staining (see also Section 5.5.3). Support for these conclusions have been provided by Wunderli et al. (1989) and D'Antonia et al. (1988) . In both cases cells containing IEA were de tected in viremic patients, frequently before the onset of viremia. This method should be applied prospectively to test its clinical utility.

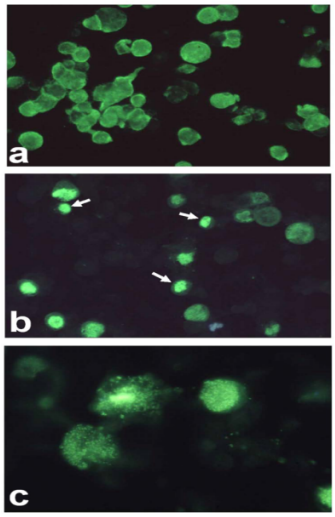

Fig1. Immunofluorescent assay. (a) RSIV-infected Grunt Fin(GF) cells were incubated with monoclonal antibody M10 and ballooned, infected cells were identified by the presence of diffuse staining throughout the cell; (b) RSIV-infected GF cells were incubated with polyclonal rabbit anti-RSIV serum which detects structural proteins. In contrast to panel A, staining is seen only within viral assembly sites. Viral assembly sites are indicated by arrows.The assembly site becomes

granulated and is gradually dispersed throughout the whole cell (Panel c);(c) Higher magnification of stained cells filled with granules.

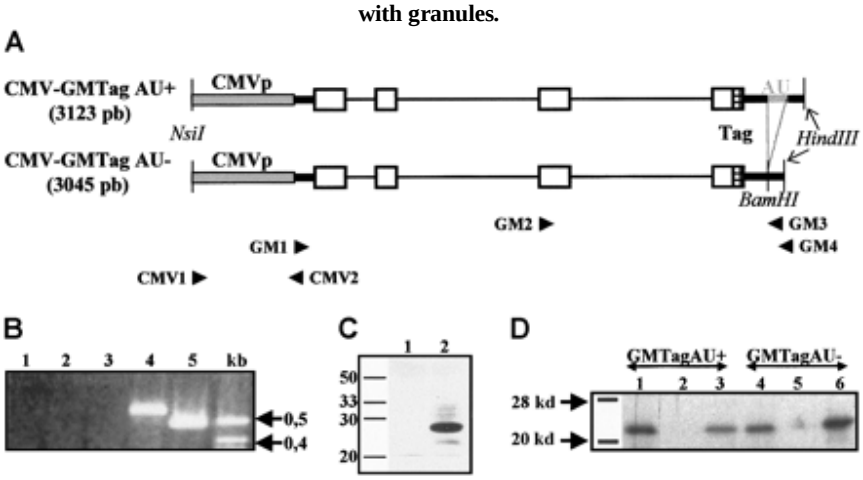

Fig2. GM-CSF gene constructions with or without ARE.

(A) Schematic representation of the CMV-GMTag AU$^+$and AU$^-$ DNA constructs. Gray box indicates CMV promoter; thick black lines, 5′ and 3′ UTRs; thin lines, introns; open boxes, coding region; gray thick line, ARE; and hatched box, c-Myc Tag. Primers used for PCR or RT-PCR and relevant restriction sites are indicated. (B) RT-PCR products derived from RNA of L-929 cells untransfected or transfected with both constructs. Lane 1, untransfected L-929 cells; lane 2, L929 cells treated with cycloheximide; lane 3, L929 cells stably transfected with pBK-CMV–GMTag AU$^+$; lane 4, pBK-CMV–GMTag AU$^+$transfected L-929 cells treated with cycloheximide; lane 5, L929 cells stably transfected with pBK-CMV–GMTag AU$^-$. (C) Detection of GMTag by Western blot; 15 µL 30 × concentrated supernatant of nontransfected (lane 1) or pBK-CMV–GMTag AU$^-$ transfected HeLa cells was loaded on 15% SDS-PAGE and blotted on nylon membrane. The membrane was incubated with goat antimouse GM-CSF primary antibody and HPR-coupled rabbit antigoat secondary antibody and revealed by ECL. (D) Translation of in vitro transcribed GM-Tag AU$^+$ and AU$^-$ mRNAs in rabbit reticulocyte lysate. After translation, 10 µL of the reaction was immunoprecipitated with anti–GM-CSF or anti-Tag antibodies. The immunoprecipitated products were analyzed by SDS-PAGE and autoradiography. Lanes 1, 2, and 3, GMTag AU$^+$ RNA translation product immunoprecipitated with antimouse GM-CSF antibody (lane 1), anti-Tag antibody (lane 3), or no antibody (lane 2); lanes 4, 5, and 6, GMTag AU$^-$translation product immunoprecipitated with anti–GM-CSF (lane 4), anti-Tag (lane 6) antibodies, or no antibody (lane 5).

Table 1 - Clinical and laboratory findings, day of onset and N-PCR for HCMV or HHV-6 in liver transplantation patients.

Pt#	Clinical and Laboratory Findings	Onset[a]	Active Infections ([a]day) by N-PCR			Disease
			HCMV	HHV-6	Co-infection	
1	Absent	-	+(91)	-	-	No
2	Absent	-	+(66)	+(87)	+	No
3[b]	Fever, granulocytopenia	76	+(181)	-	-	Yes
4	Granulocytopenia, thrombocytopenia	19	+(19)	-	-	Yes
5[b]	Granulocytopenia, thrombocytopenia, diarrhea	21	+(20)	-	-	Yes
6[b]	Fever, diarrhea	93	+(7)	+(300)	+	Yes
7[†,b]	Fever, thrombocytopenia, granulocytopenia,	127	-	-	-	No
8	Granulocytopenia, thrombocytopenia, diarrhea, fever, mucosal ulcers	30	+(25)	-	-	Yes
9	Diarrhea, poliatralgia, myalgia, liver abscesses, colonic ulcers	45	-	-	-	No
10	Absent	-	+(18)	-	-	No
11	Fever, encephalitis	24	-	+(12)	-	Yes
12	Diarrhea	-	-	-	-	No
13[b]	Diarrhea	26	-	-	-	No
14[†]	Absent	-	-	+(98)	-	No
15[†]	Fever, granulocytopenia	84	+(62)	+(62)	+	Yes
16	Absent	-	-	-	-	No
17	Absent	-	-	+(47)	-	No
18	Absent	-	-	+(6)	-	No
19[†]	Absent	-	-	-	-	No
20[†]	Absent	-	-	+(34)	-	No
21[†]	Thrombocytopenia, encephalopathy, fever	9	-	+(12)	-	Yes
22	Absent	-	-	-	-	No
23	Absent	-	-	-	-	No
24	Fever, headache	25	+(26)	+(32)	+	Yes
25[†]	Granulocytopenia, thrombocytopenia	-	-	+(77)	-	Yes
26	Granulocytopenia, thrombocytopenia	-	+(92)	-	-	Yes
27	Absent	-	+(114)	-	-	No
28	Thrombocytopenia, encephalopathy	15	+(51)	-	-	Yes
29	Absent	-	-	-	-	No
30	Absent	-	-	+(51)	-	No

Pt# = patient numbers; [a]day = post-transplant; [b]rejection; [†]death.

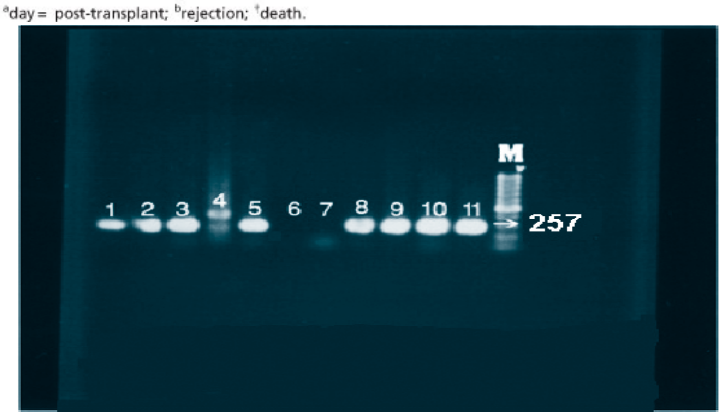

Fig 3: Ethidium bromide-stained agarose gel electrophoresis: identification of CMV DNA with p1 and p2 primers. Lane M,100 bp DNA marker (Sinagen, Iran) lane 1, positive control lane 2,3,5,8,9,10 and 11, positive patient samples lane 4 and 7, negative patient samples lane 6, negative control. The size of gB product is 257 Bp.

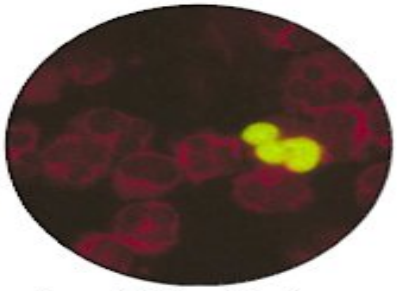

Figure 4 CMV pp65 antigens detected in nuclei of peripheral blood neutrophils

CONCLUSION AND DISCUSSION

The study of CMV infection in cell cultures is important for the detection of virus in clinical samples and for the quantitative assay of the virus. Infections in cell cultures may also be a model for the study of infection in the host (15). This is particularly applicable to the problern of persistence and latency of CMV. These terms are defined operationally in this book as follows. L ytic infection indicates infections in which complete virions are produced during a lytic replicative cycle and cells are destroyed while exhibiting cytopathology. Persistent infection indicates the occurrence of lytic infection and virus production in a cell culture, but where cytopathology is incomplete and there is simultaneously enough cell regeneration for the culture to survive indefinitely (16). Abortive infections indicate infections in which no virions are produced and the virus replicative cycle is not completed. Abortive infection may be a feature of latent infection. Some viral genes, for example, early antigens, may be expressed. Latent infection indicates that cells are infected without production of virus (17). There may or may not be transcription or translation of some of the viral genes. An essential component of latently infected cells is that if they divide, the virus genome is carried from cell generation to cell generation. In this section we also discuss the permissiveness of cells to CMV depending on the species origin of cells, their phenotypic function, and the role of cell differentiation and agents that enhance differentiation (18).

lnfection by human CMV is not strictly restricted to cells of human origin. Although viral replication has not been reported in nonhuman cells, CMV will produce abortive infections in many such cells. Fioretti et al. (1973) reported that CMV produced a cytopathic effect in guinea pig cells. A similar phenomenon in bovine and Vero (simian) cells was found by Waner and Weller (1974) (19). The cytopathic effect appeared early, 5-34 hr after infection. Refractile cells, cytoplasmic inclusions, but no nuclear inclusions were noted. Both nuclear and cytoplasmic viral antigens were found by immunofluorescence. Infectious virus was not produced unless, as in the case of infected Vero cells, they were cocultivated with human fibroblasts (20).

Monocyte differentiation or activation has been clear1y re1ated to permissiveness to CMV. Brautigam et al. (1979) observed that mouse macrophages activated with thiog1yco1ate were more permissive to murine CMV (see Section 16.6.2.2).

Weinshenker et al. (1988) studied four different human leukocyte cell 1ines. On1y one line (HUT 102) was susceptib1e, and on1y IE gene products were produced. However, a monocyte line (THP-1) was rendered fully permissive to CMV after being treated with 12-0-tetradecanoyl phorbol-13-acetate (TPA). The TPA induced differentiation so that 90% of cells became adherent, and cells proliferated, enlarged, and developed vacuo1es. Complete virions were demonstrated by electron micrographs, and 1ate antigens were demonstrated (21).

Nelson et al. (1982) identified a fragment of DNA from strain AD169 CMV that transformed NIH 3T3 and rat embryo cells so that they developed "anchorage" independence. The transformants were able to form colonies under 1.2% methylcellulose and tumors in athymic Balb/c nude mice. Additionalexperiments with deletion mutants of this fragment permitted identification of the minimum size fragment required to initiate transformation (Nelson et al., 1984). To delineate the boundaries of the transforming region pcm 4000, deletion fragments were constructed by digestion with exonuclease III and Sl nuclease, recombined in plasmids, and used to transfect primary rat cells. The results indicated that the left-hand boundary of the smallest transforming fragment must be between 490 bases (pcm 4115) and 318 bases from the Hind III site of pcm 4000. Clanton et al. (1983) identified in the Towne strain of CMV a Towne Xbal fragment E that was capable of transforming Syrian hamster embryo cells and causing tumor formation of NIH 3T3 cells. This fragment lacked homology with the transforming fragment described above but was homologous to Bg/11-c transforming fragment of type 2 herpes simplex DNA (Jariwalla et al., 1980). When fragment E was further digested with Bam HI and assayed by focus formation on NIH 3T3 and Rat-2 cells, transforming activity was localized within the terminal fragments EJ and EM (El-Beik et al., 1986). Virus-specific EM sequences were detected at less than one copy per cell in fragment E transformed cells. In contrast, EJ sequences and AD 169 transforming sequences are not retained in cells transformed by these fragments (22).

Jariwalla et al. (1989) studied the interaction between the EJ and EM fragments. Large transformed foci in Rat-2 cells were induced at a tenfold higher frequency by EJ plus EM than by either DNA fragment alone. Focus-derived cell lines also produced tumors in rats at a faster rate than lines transformed by EJ or EM alone. EJ is not detected in the transformants, but it encodes a

majorimmediate early 72-kDa (lEI) protein implicated in transactivation and autoregulation. This protein or any other protein coded by this fragment is not expressed in the transformants (18).

In summary, three transforming domains have been mapped in human CMV DNA, one a 558-base-pair fragment of strain AD169 (pcm 4127) and two located in Xbal fragment E (EM and EJ) of strain Towne. As yet there is inadequate evidence that CMV or any one of these fragments plays a role in human rliseases by transforming cells in vivo or playing a role in cancer (14).

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