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THE PREVALENCE OF HUMAN CYTOMEGALOVIRUS (HCMV) INFECTIONS IN THE OTTAWA-CARLETON REGION

by

Faten S. Gazzaz

A thesis submitted to the School of Graduate Studies in partial fulfillment of the requirements for the degree Master of Science.

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I thank my dear parents, my dear husband and my three little angels for their patience and support.

SUMMARY

In view of the low incidence of cytomegalovirus (CMV) infections in renal and bone marrow transplant patients in the Ottawa-Carleton region, a study was undertaken to assess the local epidemiology of CMV infections. First, we studied CMV viruria in age groups from 0-80 years. Secondly, we compared the isolation results obtained in the previous study with the antibody prevalence rates obtained from a retrospective serological study in the same age groups. Thirdly, we investigated CMV excretion in cervical secretions in normal pregnant women in the third trimester of pregnancy. Nineteen strains of CMV were isolated in age-groups 0-9 years. No isolate was obtained from cervical swabs of pregnant women. The serological study demonstrated a low prevalence of CMV antibodies until the age of 30. The risk of acquisition of CMV infection is low in the Ottawa-Carleton region.

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1. INTRODUCTION

Human cytomegaloviruses (CMV) are ubiquitous agents that commonly cause subclinical or inapparent infections throughout the world (136).

Mortality from CMV infections is rare and the cause would usually be recognized only as a result of special virological, serological or histological examination. Death rates therefore give no indication of the frequency of this disease (31). In terms of morbidity (31) the clinical syndromes associated with CMV infections are not distinctive and usually diagnosed only through laboratory procedures. No estimate can be made through official health records. Furthermore, most CMV infections are asymptomatic. Incidence data have come from serological and/or virological studies of groups at special risk such as pregnant women, neonates, individuals undergoing transfusions or organ transplants and heterophile-negative patients with a mononucleosis-like syndrome (31).

In seroepidemiological surveys conducted by the complement-fixation tests, the prevalence of complement-fixing antibodies is especially high were there is crowding and where living conditions are substandard. Numazaki et al (85) reported that over 60% of infants between 6 and 12 months of age at Sendai in Japan have complement-fixing antibodies to CMV. In a study done by Deibel et al (17) in Albany, New York, serologic evidence was obtained that over 90 percent of the population acquire a CMV infection during their lifespan.

The incidence of congenital CMV infection as determined in a number of large scale studies by culturing newborns' urines

varies from 0.5 to 2.2%, averaging 1 percent of all live births per annum in the United States (116). Thus, CMV is the most common of human fetal infections. The majority of these infected infants show no clinical signs at delivery (91). About 10% of these infections (or about 1 per 1000 births) result in some overt congenital CMV disease at birth, but some of the asymptomatic infections may produce effects detectable only later in life (40).

The major complications of congenital CMV infections are sequelae involving the central nervous system. Blindness, sensorineural bilateral deafness, spastic quadriplegia and hypotonia are common manifestations in severely affected infants (42). Psychomotor disturbances may range from a vegetative state with no meaningful communication to milder abnormalities of speech, behaviour and motor coordination (42).

CMV may be recovered from many body fluids or from tissues obtained by biopsy or at autopsy. The virus has been isolated from saliva (90), breast milk (44, 120), urine (90, 129), cervical secretions (84, 116), semen (70), feces (13), the upper respiratory tract (88) and circulating leucocytes (138).

This virus is probably transferred by intimate contact with an infected individual, by infusion of blood from an asymptomatic donnor, or by organ transplant.

All clinically significant HCMV infections are associated with viruria despite the fact that none of them produces symptomatic urinary tract infection or renal failure (48). CMV mononucleosis is characterized by fever, chills, myalgia, sore

throat, headache, anorexia and abdominal pain has been noted in patients 2 to 4 weeks after receiving blood transfusion. This condition is referred to as post-transfusion mononucleosis (42, 121).

Thrombocytopenia has been described in high risk premature newborns infected as a result of blood transfusion. A mononucleosis-like illness has been described after transfusion of large amounts of blood in open heart surgery (121).

Many patients who have undergone renal transplantation become infected with CMV following surgery, particularly seronegative patients receiving a kidney from a seropositive donor. Patients with preexisting antibody may become infected but are often asymptomatic.

CMV infections are frequent and occasionally severe in children or adults with congenital or acquired defects of cellular immunity (42). These are patients with cancer, particularly leukemia (45) and lymphoma (36) and those on treatment with immunosuppressive drugs (36).

Disseminated CMV infection with involvement of the lung, liver, spleen, adrenal glands, myocardium and gastrointestinal tract has been found on autopsy in one to three percent of children with leukemia (121).

Acquired CMV infection has also been associated with hemolytic anaemia and thrombocytopenia (43), ulcerative lesions of gastrointestinal tract and bleeding (10).

So, although frequently inapparent or asymptomatic, the incidence and spectrum of disease in newborns and in

immunocompromised hosts establish this virus as an important pathogen (121).

It is often assumed that epidemiological data gathered in other parts of North America apply to the Canadian situation. The following are examples of some recent U.S. data:

- Ho et al 1975 (47) determined that 66% of renal transplant recipients showed infection with CMV.
- Chatterjee et al 1978 (11) reported from the University of California that 91% of renal transplant recipients were infected by CMV.
- Meyers et al 1980 (82) reported from the University of Washington that 58% of bone marrow transplant recipients were infected by CMV.

Preliminary data obtained suggest that the situation is different in the Ottawa-Carleton region and this has important implications in terms of prevention of CMV infections:

a) Phipps and Rossier (95) studied the prevalence of antibodies against CMV among 717 healthy, unselected pregnant subjects seen at the Ottawa General Hospital for routine pregnancy examination. Only 34.9 percent were seropositive to CMV by the complement-fixation test.

b) At the Regional Virology Laboratory for Ottawa-Carleton, patients undergoing renal and bone-marrow transplants have been monitored for CMV both virologically and serologically, since 1982.

Out of 13 bone-marrow transplants, 2 seroconverted (15.4%) for CMV and no virus was isolated from their urines (0%). Out of

38 renal transplant recipients, 14 seroconverted (37%) for CMV and the virus was isolated from the urines of two patients (5.5%).

c) Over the past ten years, out of four possible clinical cases of congenital cytomegalic inclusion disease admitted to the Children's Hospital of Eastern Ontario, none of them was laboratory confirmed either serologically or by isolation of the virus.

This is a remarkable finding considering that every single neonate with any suspicion of congenital infection is systemically investigated serologically including IgM detection and isolation of CMV from the urine.

The general objective of our research was to study the prevalence of HCMV in our region, with the following objectives:

- To determine the frequency of CMV urinary excretors in age groups from zero to eighty years of age.
- To carry out a retrospective serological study of the same age groups as above and to compare these data with the isolation findings.
- To study the rate of cervical CMV excretion in healthy pregnant women during the third trimester of pregnancy.

2. LITERATURE REVIEW

2.1. Characteristics of HCMV

2.2. Epidemiology of HCMV

2.3. Isolation of HCMV

2.1. Characteristics of Cytomegalovirus:

2.1.1 Nomenclature and classification:

Human cytomegalovirus (HCMV), a member of the herpes virus group, was first isolated in 1956 (114).

In 1979, the International Committee for the Nomenclature of the Viruses (ICNV) proposed that the family of herpesviridae be divided into three subfamilies representing:

The herpes simplex viruses (Alphaherpesvirinae)
cytomegaloviruses (Betaherpesvirinae) and
lymphoproliferative virus groups (Gammaherpesvirinae).

2.1.2. Main characteristics of the HCMV group:

The DNA molecular weight is 150 x 10^6. The virus is recovered only from human infections. The CMV group has a relatively slow reproductive cycle (>24 hr), forming slowly progressing foci in cell cultures. Enlargement of the infected cell occurs in vivo and often in vitro (cytomegalia) (77). Inclusion bodies containing DNA may be present in the nuclei and the cytoplasm late in infection (77). Latent infection is frequently present in the salivary gland and/or other tissues (77).

The host range is narrow, in cell culture, HCMV usually grows best in human fibroblasts, and less well in certain human lymphoblastoid cells (77). McFarlane and Sommerville (76) have reported the growth of HCMV strains in Vero cells.

2.1.3. The structure of HCMV

2.1.3.1. The virion

The gross structure of HCMV resembles that of herpes simplex virus. The nucleic acid core is surrounded by a capsid, about 110nm in diameter. The capsid has an icosahedral structure; it consists of 162 hollow, elongated polygonal capsomeres, and an outer lipoprotein envelope (113). Some of the particles are surrounded by an envelope consisting of a single or double membrane. Many of the particles have the loose outer envelope characteristic of the herpesvirus group, which is readily inactivated by lipid solvents and relatively unstable at room temperature.

In virus-infected cells, virus particles are found within the nucleus (intranuclear inclusion bodies). They consist of a central core, about 60nm in diameter, surrounded by a single membrane (5). A second coat is acquired from the inner nuclear membrane as the particles migrate to the cytoplasm. The cells explode with a mushrooming effect (68) and the virus is transferred from cell to cell. Electron microscopy analysis (112) of the virus replication reveals that virions and dense bodies (they are described in 2.1.3.3.) enter the cell either by fusion of the envelope with the cell membrane or are uncoated following phagocytosis.

2.1.3.2. DNA of the HCMV

Viral DNA is only made in the nucleus, presumably viral DNA in the cytoplasmic inclusions migrates from the nucleus (48). It

is double stranded. The molecular weight of the DNA of HCMV is 130-155 x 10^6 (62) with a base composition (G + C) of 57 to 74%. Its DNA is about 7% of the particle weight (48). It consists of an L and an S component which are covalently linked (62). The L and S components have molecular weight of approximately 124 x 10^6 and 26 x 10^6 respectively. Each component may be inverted leftward and rightward (52). This gives four possible arrangements of the DNA molecule. Sequences from either or both termini may be present in an inverted form internally (77).

Comparison of strains at the level of nucleic acid homology reveals that the human CMV isolates share at least 80% of their DNA sequences by comparison with AD 169 strains (49). However, when human isolates of CMV are compared by restriction endonuclease analysis they are readily distinguishable (51).

With regard to CMV and herpes simplex viruses, characterization of their DNA by restriction endonuclease analysis is the most practical and discriminating means of determining genetic relatedness between different isolates (50). There are many restriction endonuclease enzymes, originally purified from bacteria, that uniquely cleave DNA at different sites (102). The resultant fragments of DNA differ in number, size and chemical composition and therefore migrate at different rates on electrophoresis in agarose gels. The development of the gel patterns, or fingerprints, depends on the enzyme used and the structure of the DNA under examination. Through comparison of the DNA fingerprints of different viral isolates with use of two or more restriction enzymes the degree of genetic relatedness can

By DNA - DNA reassociation kinetics or nucleic acid hybridization there is about 80% homology among different CMV strains (49), while the degree of homology existing among HSV type 1 and type 2 is about 47 - 50% (61).

2.1.3.3. Dense body proteins

Dense bodies are homogeneous electro-dense materials, two to three times the diameter of enveloped virus (48). Dense bodies are aggregates of virus substructures that share an envelope and are abundant in the cytoplasm of infected fibroblasts (48) or they are characteristic intracytoplasmic accumulations of viral structural proteins. How they differ from virions in composition is still controversial.

Sarov and Abady (105) separated dense bodies from virions by centrifugation in 30-60% (w/w) sucrose density gradients. By SDS urea polyacrylamide gel electrophoresis (PAGE), they identified 23 proteins ranging from 24,500 to about 171,000 daltons. Dense bodies and virions were similar in composition, excepted that polypeptide VP 18A was not detectable in dense bodies, and five other polypeptides were barely detectable. The major protein of dense bodies was VP_9 (m.w. 67,000), which accounted for 40% of the total protein complement, whereas it accounted for only 20% of the virion protein complement.

Labelling of the DNA of infected cells showed that dense bodies contain no DNA (105).

A significant finding was that by immune electron microscopy, specific antibody clumped both virions and dense bodies. Hence, the membranes of dense bodies possessed viral antigens (48). This view has been substantiated by more recent biochemical findings that dense bodies and viral capsids are composed of essentially the same structural proteins (105, 25, 63, 64). The relationship of dense bodies and virions to lysosomes has now been further clarified in a recent electron microscopic study by Smith and DeHarven (112). Dense bodies are not lysosomes but enveloped capsids. Naked capsids, as well as dense bodies, acquire an envelope by budding directly into lysosomes which contain acid phosphatase and arylsulfatase. Dense bodies are formed in proximity to microtubular membranes and the Golgi apparatus (48).

2.1.3.4. Cytoplasmic inclusions

Cytoplasmic inclusions were early noted to be a hallmark of CMV infection (48). Cytomegalovirus produces perinuclear cytoplasmic inclusions, a unique feature among herpesvirus (48). They are homogeneous masses of dense bodies, virus particles and microtubules (48).

2.1.3.5. Defective interfering particles (D.I.) and their DNA

Von Magnus (1947) (131) was the first to show the presence of incomplete type A influenza virus (D.I.) in relation to multiplicity of infection.

D.I. particles are incomplete forms of virus apparently derived with surface structures similar to the complete virus. These forms are pleomorphic in appearance. The characteristic central structure is usually missing as a result of failure in the final assembly of the nucleoprotein core into the shell provided by the cell membrane (27). They may account for the attenuation of viral virulence or the persistence of certain virus infections (48). The usual method to produce D.I. particles is by high multiplicity passage of a virus strain in an animal host or in a permissive cell culture system (48).

In case of HCMV, restriction enzyme analysis of the D.I.'s showed that these DNA molecules were different from the DNA of complete particles (127). Stinski et al showed that the majority of D.I. DNA molecules had a molecular weight of approximately 100 x 10^6, while DNA molecules associated with low multiplicity passaged HCMV (Town strain) had a molecular weight of approximately 150 x 10^6 dalton.

2.1.3.6. Protein synthesis and viral replication

HCMV does not completely shut off host protein synthesis. Although CMV takes 72 h or longer to produce progeny virus, interesting biochemical events occur much earlier (48) (Table I)

Event	Hours after infection
Immediate early protein synthesis	0 - 4
Early protein synthesis	2 - 20
Induction of DNA polymerase	20
Stimulation of cell DNA	20
Viral DNA synthesis and late progein synthesis	15 - 72
Synthesis of IgG receptors	36

TABLE I: Biochemical Events in CMV replication (48)

Stinsky (1976) (124) tried to isolate membrane glycoproteins of virions and dense bodies. Eight glycoproteins were repeatedly detected and three occasionally.

Stinski (1977) (125) analysed structural proteins of purified HCMV of 9% slab polyacrylamide gels. A minimum of 35 polypeptides were identified. Virions and dense bodies shared 24.

According to Stinsky (1978) (126) protein synthesis following CMV infection may be divided into two stages. There is an early stage with a peak of synthesis that is predominantly (70-90%) host specific, although some specific viral proteins are also made. Specific viral DNA synthesis initiates a second stage of protein synthesis which is predominantly viral, but 40-50% of the protein synthesized is still of host origin.

The early phase of virus specific protein synthesis can further be divided into "immediate early" and "early". The "immediate early" group is the first group made, usually within 4 hours after infection. This group is assumed to play an important role in modulating subsequent protein synthesis, and

includes specific early antigens found in immuno-fluorescent tests (32). Late protein synthesis in CMV infected cells is proceeded by DNA synthesis and can be aborted if DNA synthesis is blocked by phosphonoacetic acid. All viral structural proteins are synthesized in this second stage (125).

2.1.4. Stimulation of cell DNA and RNA

Because CMV produces latent infections, much interest was evoked by the finding that CMV stimulates cellular DNA synthesis and can transform cells. Infection of cells by CMV, even at high multiplicity of infection, causes an increase in host cell DNA, RNA, and protein synthesis (125). The possible pathways of CMV genome in an infected cell are shown in figure 1 (128).

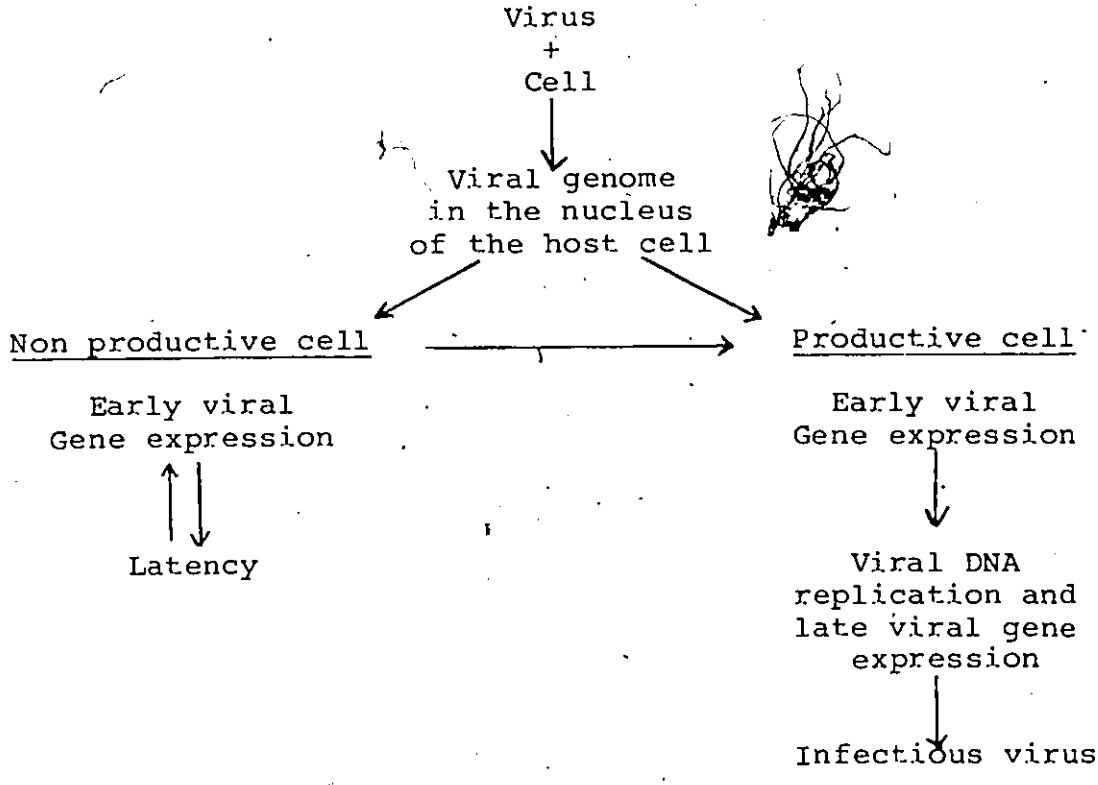


FIGURE 1: Proposed pathways of the CMV genome in non-productive and productive cells.

It is implied that CMV encounters both non productive and productive cells in the human being, the type of pathway may be influenced by the physiology of the cell at the time of infection. It is proposed that early viral gene expression occurs in both types of cells. Latency occurs in the non productive cell when the genome does not proceed to replication. It is also proposed that physiological changes or the eventual expression of a required early viral gene can convert the non productive cell to a productive one and, consequently, the viral genome can go from a state of latency to replication and production of infectious progeny.

2.1.5. Stability of Cytomegalovirus

The extracellular virus is inactivated rapidly at 37°C (137) but certain diluents, such as distilled water, appear to have some stabilizing effect on the virus at this temperature (137). Urine specimens were stored for several days at 4°C without appreciable loss in titer (23,72). When stored for several months however, the infectivity titers decreased. Storage of CMV at -20°C results in complete loss of infectivity within a few days (23). CMV could be preserved satisfactorily by quick freezing and storage period at this temperature. Addition of 25-35% sorbitol delays the complete loss of infectivity for a few months if added to stored CMV at -20°C. In transport medium (TM) the inactivation of CMV is delayed for several days. Virus in water maintained its titer for 7-10 days without reduction. The salt concentrations and PH of suspending medium were clearly important. HCMV is inactivated at PH 3. It is sensitive to diethyl ether and chloroform.

2.1.6. Antigenic Structure

Antigens eliciting antibodies of the IgG, IgM and IgA classes have not been precisely defined. However 2 complement-fixing (CF) antigens have been described (132, 65, 124). These 2 antigens can be separated from the virus particles by differential centrifugation, and it seems probable that the soluble antigen is a structural protein of the virus released by disintegration of the virus particle (7). The soluble antigen contains at least 2 polypeptides with molecular weights of 66,000 and 140,000 daltons. The smaller is glycosylated and hyperimmune animal serum prepared against it does not neutralize homologous virus (132). Soluble antigen is stable at 4°C and withstands repeated cycles of freezing and thawing but marked loss of potency occurs at 37°C or with boiling (65). The virus associated CF antigen extracted by glycine buffer treatment of infected cells appears to derive its antigenicity primarily from nucleocapsids (34). Both antigens develop primarily after viral DNA replication and are mostly cell associated. Supernatant fluids from infected cell cultures contain minimal amounts of CF antigens.

It was reported that HCMV strains share a common antigen which may be readily detected in the complement fixation test (68). It has been shown that there were no significant differences in the antibody titers detectable in a given human serum tested against the antigen of the three prototype strains of human CMV, including Davis, AD-169 and Kerr/Epaillat strains using the complement fixation techniques (67).

The antigens eliciting neutralizing-antibody production are found in the envelopes of mature virions and dense bodies and are likely glycoproteins (124). Antiserum prepared against purified virion and dense body membrane glycoproteins neutralize infectivity and react with cytoplasm and membranes of infected cells in indirect-fluorescent antibody (IFA) tests, yet elicit no fluorescence of the membranes of uninfected cells (130).

It was reported that there is much less homogeneity among HCMV strains investigated by means of the neutralization test (NT) than complement fixation test (135), and that the specificity of CF reactions is usually broader than that of neutralization or hemagglutination inhibition (HI) test (135).

Pereira et al (94) reported extensive analysis of 15 strains of CMV, including the Towne strain, in immunofluorescence tests with a panel of monoclonal antibodies to the surface proteins of CMV infected cells and showed that all of the strains specified cross-reactive antigenic determinants. The monoclonal antibodies used in this study identified antigenic determinants strongly conserved among different strains in nature.

There are several implications from the above for the serological diagnosis:

Following the report of Weller et al (135), numerous attempts to improve the serological diagnosis of CMV infection by using many types of antigens did not improve sensitivity. From the point of view of specificity it was reported that neutralization titers were low and frequently absent, whereas CF titers were always positive and diagnostic (78), but NT is the

most strain specific. Neutralizing antibodies function against a small number of antigens on cell surface which are important to the attachment of the virus to the cell.

There are therefore antigenic variations among CMV isolates, but these are not great enough or clear enough to warrant the designation of different types of human CMV.

However, the important question, as to how much cross immunity there is in terms of resistance of the host to other strains of HCMV following a primary infection, remains unresolved.

2.1.7. Clinical aspects

There are 2 forms of infection: congenital or acquired.

2.1.7.1. Congenital infections

Approximately 95 percent of infected infants are asymptomatic at birth. In large surveys of infected newborns followed for several years after birth, about 20 percent have been found to be significantly affected by congenital CMV infection (42).

The clinical manifestations are, in order of decreasing frequency, hepatosplenomegaly, jaundice, purpura, microcephaly, chorioretinitis and paraventricular cerebral calcifications. A petechial rash on the first day after birth is suggestive of CMV infection. Often the only finding is general failure to thrive or increased irritability. Some infants have repeated respiratory infections. The frequency of congenital abnormalities in infants with CID is increased, these include clubfoot, inguinal hernia, strabismus, high arched palate, microcephaly and deafness (42).

2.1.7.2. Acquired infections

Here we are concerned with the clinical aspects of CMV acquired infections, confirmed by urine and/or throat cultures for CMV (48).

The perinatal infection may cause symptomatic infection such as pneumonitis, but it is a fairly rare event in probably less than 1% of HCMV perinatal infections. One can assume that the normal neonate, unlike the embryo, is quite resistant to CMV disease, as are the child and normal adult. A perinatal primary infection acquired by blood transfusions is a clear exception to the above statement (48).

The acquired infection during infancy and early childhood is usually inapparent, although respiratory symptoms occasionally occur, with pneumonia, paroxysmal cough, petechial rash, hepatomegaly, and splenomegaly. CMV associated myoclonic seizures in infants and infectious polyneuritis and encephalitis as well as chorioretinitis in adults have been reported. Hepatomegaly and mildly abnormal liver function tests are found in asymptomatic individuals excreting CMV in the urine and also in patients with CMV mononucleosis. The latter may experience malaise, anorexia and abdominal pain (42). The disease is characterized by a rather long incubation period (4 to 8 weeks) followed by viremia of a few weeks duration, with prolonged excretion of the virus from the pharynx and the urinary tract (55).

In contrast to herpes simplex and varicella zoster, which in acquired postnatal infections, are mainly dermatropic and occasionally neurotropic and to EB virus, which primarily affects the lymphatic system, CMV attacks peripheral leucocytes, lymphoid and glandular tissues and visceral parenchymatous organs (17).

Unlike EBV induced mononucleosis, exudative pharyngitis and prominent cervical adenopathy are less typical of CMV induced disease which is responsible for approximately 70% of cases of heterophile negative mononucleosis (87).

CMV infection in the immunosuppressed is frequently asymptomatic. When symptomatic, a febrile mononucleosis, similar to the disease in the normal adult, already described, appears to be the most common presentation in the transplant patient. The cardinal characteristic is prolonged fever. There may be also atypical lymphocytosis and mild hepatitis. After febrile mononucleosis, the next most common symptom of CMV infection is pneumonitis (48).

2.2. Epidemiology of HCMV

2.2.1. Transmission and excretion of HCMV

Several mechanisms are probably responsible for the transmission of CMV from one host to another depending upon age, state of host resistance, medications and other treatment such as blood transfusions and hemodialysis. Transmission from person to person requires intimate contact and may occur by percutaneous or permucosal contact with tissues, secretions or excretions of infected persons (40) and can be transmitted from mother to child. Prenatal transmission through placenta has been

demonstrated by Stagno et al (115). Intrapartum transmission was suggested by Numazaki et at (85). CMV excretion in human milk has been reported by Hayes et al (44). Lang and Kummer suggested venereal transmission after puberty (70). Montgomery et al (84) isolated CMV from cervical swabs. The respiratory route is suspected as another route of transmission (88).

2.2.2. The effect of age

The epidemiology of CMV infection during the first year of life is quite different from that in other periods of life (85).

Table II lists the frequency of viruria at various times during the first 2 years of life as an index of perinatal infection.

Age affects virus shedding. Viruria and respiratory shedding are relatively frequent during the perinatal period. They may occasionally be found during childhood, but viruria is rare in healthy adults. Interestingly, shedding decreased steadily with age (48).

Duvall et al (20 were unable to isolate CMV from 17 healthy adults contacting an infected patient.

Stern (122) found a single CMV excreter among 101 children 5 to 9 years old. The author was unable to recover CMV from 474 adults in the age-group between 15 and 35 years.

2.2.3. Prevalence of antibodies in the general population

The prevalence of antibodies to CMV varies with the socioeconomic status of the population studied.

The relevant data in population groups over the age of 35 were summarized by Krech (69) (Table III). The same diagnostic reagents and technical procedure were applied by all the participating laboratories and sera were tested at a dilution of 1:4. The prevalence is lower in Europe, Australia and parts of North America, whereas it is significantly higher in "less developed" areas. Some regions with warmer climates such as Uganda, the Phillipines and Tanzania have a prevalence of 100%. It is however, unlikely that warm weather is a decisive factor, since prevalence among Eskimos is also high (81%) (111).

TABLE II: Frequencies of CMV Viruria during the First 2 Years of Life[a]

Authors	Locality	Total No. of infants studied	Percentage excreting CMV according to age (months)							
			At birth	1	2	3	6	9	12	24
Numazaki et al. (1970) (85)	Japan	257	-[b]	6	10	20	56	44	22	7
Olson et al (1970) (88)	Thailand.	140	-	-	-	38	55	18	15	-
Granstrom et al. (1977) (33)	Finland	148	2	-	16	32	36	-	39	-
Ahlfors et al. (1978) (1)	Sweden	326	1	12	-	-	-	-	23	-
Levinsohn et al. (1969) (74)	U.S. Seattle	92	1	3	-	11	13	-	11	-
Stern (1968) (122)	England	118	2.5	-	-	-	9	-	-	-

[a]Adapted from Stagno et al. (1980) (70)

[b]-, No data available

City	Country	Percent with CF antibody
Lyon	France	40
Freiburg	Germany	42
St. Gallen	Switzerland	45
Albany	U.S.A.	45
Merbourne	Australia	54
Houston	U.S.A.	79
Buenos Aires	Argentina	81
Hong Kong	China	94
Sandai	Japan	96
Manila	Philippines	100
Morocco	Morocco	98
Entebbe	Uganda	100

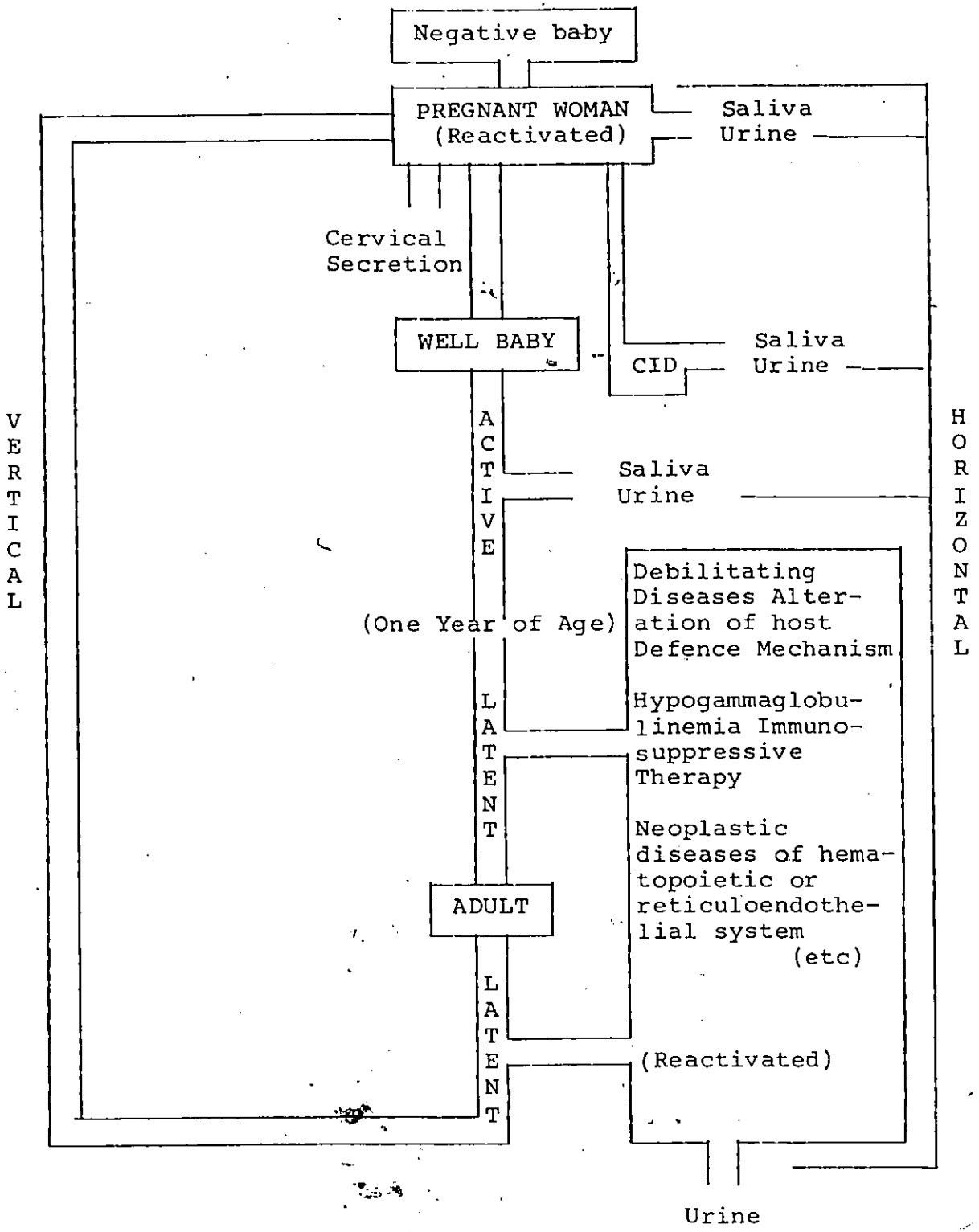
TABLE III: Prevalence of complement fixing antibodies in various regions of the world (69). Sera were screened at a dilution of 1:4.

2.2.4. Epidemiology of HCMV in different groups

Figure 2 represents a model of the epidemiology of CMV as proposed by Numazaki et al (85). Their hypothesis is that CMV is perpetuated by a vertical and horizontal transmission cycle as illustrated:

1) When pregnant women are infected exogenously with CMV in early pregnancy CMV can be transmitted transplacentally to fetus and results in CID (cytomegalic inclusion disease).
2) Healthy infants excrete the virus into saliva and urine.
3) After one year of age, these healthy infants become carriers of latent infection.
4) By the administration of corticosteroid (20) or by immunusuppressive therapy (15), activated virus spreads to various organs.
5) Virus is reactivated during pregnancy perhaps due to the elevation of certain hormones (20).

FIGURE 2: The hypothesis of the ecology of human cytomegalovirus.


Negative baby
PREGNANT WOMAN
(Reactivated)
Saliva
Urine
Cervical
Secretion
WELL BABY
CID
Saliva
Urine
VERTICAL
ACTIVE
Saliva
Urine
HORIZONTAL
Debilitating
Diseases Alter-
ation of host
Defence Mechanism
(One Year of Age)
LATENT
Hypogammaglobu-
linemia Immuno-
suppressive
Therapy
Neoplastic
diseases of hema-
topoietic or
reticuloendothe-
lial system
(etc)
ADULT
LATENT
(Reactivated)
Urine

2.2.4.1. CMV INFECTION IN PREGNANT WOMEN AND THEIR INFANTS

Normal women whose serum is positive for antibody to CMV frequently shed virus from a single or several sites, including the genital and urinary tracts, pharynx and breast-milk (39, 85, 99).

Active excretion of CMV aby the pregnant women in urine is not uncommon (40). CMV is also excreted in the cervix of pregnant women with higher rates ~~in~~ late pregnancy, 1.5% in the first trimester to 13.5% in the third trimester (84, 85, 116). Many infants also become infected in the early post partum period because of exposure to a maternal or other sources of virus during or soon after delivery (118).

Virus in the cervix and urine at the time of delivery in many mothers provides a "sea of cytomegalovirus" through which the newborn infant must pass and be placed at high risk of infection (32).

CMV isolated from mothers and their children were studies by Huang et al (51) to determine whether recurrent infections and transmission to the fetus in immune women were due to reinfection or reactivation of latent endogenous virus by means of restriction endonuclease analysis of purified viral DNA (they have been described in 2.1.6.). Huang et al concluded that endogenous CMV appeared to be the most frequent source of recurrent infection and intrauterine transmission in immune women; reinfection also occurred, but less commonly. However, perinatal transmission is much more common than intrauterine transmission (33).

Infection during pregnancy may be of two types (48) the first is the usual systemic infection accompanied by a serologic rise some of which may result in persistent virus shedding in the cervix. The second is a local infection of the cervix and occasionally, of urine. The main characteristic is virus shedding without a serologic change. The pathogenesis of this type of infection is not clear, but it may represent a local reactivation of infection associated with the hormonal changes of pregnancy.

2.2.4.2. Epidemiology of CMV in infancy and early childhood,

2.2.4.2.a. Epidemiology of CMV in healthy normal children

Healthy infants living at home, from birth to one year of age were studied (85) for evidence of CMV infection in Japan. The results suggested that infection with HCMV occurred in over 60% of healthy Japanese infants living at home, without clinical manifestations, by 5 months of age.

It was reported that CMV was found in the respiratory tract of 21.2% of 378 Thai children less than 4 years of age (88) (Table II).

Cytomegaloviruria was found in 13 (72%) of 18 children between 2 and 3½ years of age in a day nursery at Stockholm, Sweden during an epidemic-free period of nearly 2 years (129).

CMV was recovered from 53 percent of urine specimens and 45 percent of mouth swabs in Birmingham Alabama, showing that 51 percent of children were excreting CMV (90). The prevalence of excretion varied according to age, with the lowest rate

9 percent) observed in infants less than one year of age and the highest rate (83 percent) among toddlers in their second year of life. A potential route of transmission in the Alabama study (90) was identified by the recovery of CMV from toys that had been mouthed by toddlers and walking children which allow close contact and sharing of toys or hand contact with infected urine due to changed diapers or unwashed hand after passing urine.

2.2.4.2.b. Role of prenatal CMV infections in mental retardation

Intrauterine infections which are at present known to be capable of causing severe brain damage include syphilis, rubella, CMV and toxoplasmosis, are commonly subclinical and unrecognized (123).

Affected infants may be born apparently well and only subsequently develop evidence of mental retardation (78). Some patients with neonatal cytomegalic inclusion disease recover completely or with slight motor or mental disability, but the majority have severe residual abnormalities due to brain damage, the most important being mental deficiency and microcephaly (78). Epilepsy, cerebral palsies, hydrocephalus, blindness and deafness may also follow.

By studying mentally retarded children (123) a significant association was found between CMV infection and microcephalic mental deficiency.

The quantity of virus excreted in the urine during early infancy was significantly greater in infants who acquired infection in utero, particularly among those born with overt

disease; thereafter, all three groups (congenital symptomatic, congenital asymptomatic and natal) excreted similar amount of virus (117).

2.2.4.3. Epidemiology of CMV infections transmitted by blood-donors

CMV infection in humans can be the result of the introduction of virus into a non-carrier by transfusion with blood from a carrier (139).

Generalized cytomegalic inclusion disease and cytomegalovirus mononucleosis have been shown to develop after the transfusion of fresh blood (57) in neonates, with renal haemodialysis (16) or large transfusions of fresh blood or surgery with extracorporeal circulation (89).

It has been reported that latent CMV infection is associated with leukocytes (138) giving the priority to donors-seronegative for CMV complement-fixation antobody as potential donors of leukocytes. Further consideration should be given to the use of leukocyte-depleted blood products in patients at high risk from CMV infection. It is thought that freezing destroys the viability of leukocytes which may be essential for carriage of the virus.

Neonates may acquire CMV infection as a result of blood transfusion (140), including exchange transfusion. Winston et al (138) observed more CMV infections in marrow transplant patients and newly diagnosed acute leukemia patients who had received a large number of leukocytes transfusions. An afebrile syndrome

with hematologic features of infectious mononucleosis has been found to occur after open heart operations in 3 to 11 percent of patients (26, 28).

A significant (eight fold or more) rise in the titer of complement fixing antibodies to the CMV has been reported in 30 percent of patients submitted to open heart operations with the use of extracorporeal circulation and fresh blood (89). In this last study, it seemed that the risk of contracting a CMV infection was greatest in patients with no demonstrable CMV antibodies or a low titer before the operation.

2.2.4.4. Epidemiology of CMV in renal transplant patients

The development of CMV infection in renal transplant patients definitely leads to an increase in morbidity (8, 47) and in some studies to an excess allograft loss (8). A major source of CMV infection appears to be the transplanted allograft or the cells trapped in the kidney (47). Transfused blood may also be a source of CMV (89). It has been also suggested that person to person transmission may occur in the dialysis unit (14). If the latter is true, then nursing and technical personnel of child bearing age might be at risk due to acquisition of CMV infection in that setting (41). The transplanted kidney could be the source of primary infection in the transplant recipient (47). On the basis of serologic tests, infection in a seropositive recipient is thought to indicate reactivation and infection in a seronegative recipients is thought to indicate a primary infection (101).

2.2.4.5. Epidemiology of CMV in bone marrow transplant patients

Exogenous infection in many bone marrow transplant patients is the cause of CMV infection (82). The source of infection may be infected blood products (19). There is indication of a lack of protection by preexisting antibody among sero-positive patients, whereas in cardiac and renal transplants with pre-existing antibodies, the infection is less severe (8, 96).

Whether recurrent infection is due to virus reactivation or exogenous reinfection, may not be valid after marrow transplant because of the unusual immunologic situation in which the donor immune system is transferred to the marrow recipient. Indeed, Meyer's study of HSV (81), varicella zoster (80) and CMV (82) infections suggest that specific cell-mediated immunity to herpesviruses after marrow transplant does not recover until the patient experiences an active virus infection. Thus all CMV infections after marrow transplant may be considered primary infections (82) and that not only the recipient's immunucytes are obliterated, but for unknown reasons, the donated marrow cells (lymphocytes) do not exhibit some types of immunologic competence, even if the donor was seropositive.

It should, however, be noted that bone marrow recipients, unlike renal transplant recipients, are severely immunosuppressed by irradiation and cytotoxic drugs prior to transplantation (48).

2.2.4.6. Sexually transmitted CMV and cervical atypia

Epidemiological studies (4, 103) have associated cervical cancer and dysplasia with sexual activity.

A relationship between herpes simplex type 2 (HSV-2) and these two conditions has been reported (4, 79, 86). Chlamydia trachomatis, Trichomonas vaginalis, papilloma virus and perhaps CMV may be able to induce atypia (106). The presence of CMV in the cervix as well as its presence and persistence in semen (70) raises the possibility of venereal transmission, and one epidemiological analysis is consistent with this possibility (56).

2.2.4.7. Acquired Immuno Deficiency Syndrome (AIDS) and its relationship to CMV

CMV infections are known to induce alterations in the immunoregulatory suppressor and helper T-lymphocyte populations and it has been suspected to be a possible cause of immuno-suppression (97).

DNA - DNA reassociation kinetics, anticomplement immunofluorescence (ACIF) and ACIF-blocking tests were used to detect CMV gene products in Kaposi's sarcoma (K.S.) biopsies and early cell cultures derived from them (30). Three of eight tumour biopsies were positive for CMV DNA from different patients. Recently it has been reported that AIDS is caused by a human T-cell lymphotropic retrovirus, termed "lymphadenopathy-associated virus" (LAV) or human T-cell lymphotropic virus type III (HTLV-III).

2.2.4.8. CMV infection in patients with malignancies

The patient with lymphoreticular malignancy or leukemia, particularly while under intensive corticosteroid therapy and chemotherapy, might be expected to (a) develop activity of viruses previously latent, or (b) present an unusual response to agents newly acquired (36). Urines and throat swabs should be repeatedly cultured to detect infection with CMV (45). It appeared to be a good correlation between excretion of virus (in urine and/or throat) and the presence of complement fixing (CF) and indirect-hemagglutination (IHA) antibody (45).

In leukemic patients, the timing and pathogenesis of CMV infection remains unclear.

Cytomegaloviruria in leukemic children receiving chemotherapy occurred with a frequency comparable to that in control groups (6, 21). However, there is a higher than expected rate of seroconversion in leukemic patients who are originally lacking CMV complement-fixing antibody (130).

2.2.5. Immunity to CMV

The production of antibodies following viral infections is almost a universal response. Antibodies are usually produced following apparent or inapparent infections, even in the severely immunosuppressed subjects, and infection is often accompanied by clinical disease despite the presence of circulating antibodies. One assumes that they are inadequate to present latent infection and disease manifestation (48). CMV can be transmitted in utero and can result in congenital infection in spite of substantial

maternal immunity (118). Breast feeding did not protect against infection (48) and CMV was found in the milk of about one quarter of women with CMV-CF antibody (44). Severe congenital infection invariably stimulates antibodies in the neonate (48).

Cell-mediated immunity is of particular interest because it is presumed to be the main host defense against CMV infections. The most impressive indirect evidence that specific CMI is indeed important in man is the frequency of CMV infection following its suppression (48).

A study by Pass et al (92) demonstrated prolonged viral shedding in children with congenital CMV infection despite the presence of serum antibody. In the latter study the symptomatic patients had significantly weaker specific blastogenic responses from one to seven years of age. The impairment was highly specific for CMV, as patients with congenital CMV infection who had antibody to herpes slimplex virus did have lymphocyte blastogenic response to the latter virus.

2.3 Identification of HCMV

2.3.1. Exfoliative cytology and histopathology

Characteristic exfoliative cytology findings include giant cells, containing large intranuclear inclusions with a well defined nuclear membrane surrounding the halo around the inclusion. Cytoplasmic inclusions may also be seen (73). The cell smears may be suitably strained by Giemsa, haematoxylin and eosin or by the Papanicolau method (24). The appearance of these cells in body fluids results from the desquamation of infected

cells and their release into urine, saliva and other body fluids (73). However, this simple method, not requiring a special laboratory, has a high rate of false negative results even in infants with classical symptoms of cytomegalic inclusion disease. For large scale screening this technique is too insensitive and time consuming to be effective (38). Only 50% of the urine samples from infants with symptomatic congenital HCMV yield positive results using histopathology (109). In addition, the presence of these cells in urine sediment is not pathognomic of HCMV, since they may be found in the urine of patients suffering from a variety of viral infections (109).

Recently, cytopathic effects of cytomegalovirus infection were studied in human cell cultures at various time intervals (2) comparing five different strains of CMV at a multiplicity of infection of approximately 5 plaques forming units per cell. CMV strain associated differences in cytopathology were found and they could result from variances in biologic characteristics of strains studied and they were independent of the severity of infection or source of fibroblastic cells.

2.3.2. Electron Microscopy (EM)

Electron microscopy has been proposed for rapid diagnosis of CMV infection, but its morphologic similarity to the other herpesviruses precludes establishing a definitive diagnosis by this method.

EM is also limited in its usefulness by its relative

insensitivity and its unsuitability for processing large numbers of specimens.

Comparative studies between EM data and inoculation into tissue culture have shown that this method was consistently reliable if the virus concentration in the urine was greater than 10^4 FFU/ml. However, it was less satisfactory if the virus concentration was lower than 104 FFU/ml, or if a distinction from other herpesvirus was required (48).

2.3.3. Identification of CMV in cell cultures

So far, the current method used to diagnose HCMV infection is by virus isolation. Urine supernatant can be inoculated directly in human fibroblasts cultures only (described in 2.1.2.3.) (73).

Current tissue methods take from less than one up to seven weeks to show any cytopathic effect, which is a slow procedure in which results are often obtained too slowly to be useful in diagnosis and management.

Characteristically, the cytopathic effect (CPE) induced by clinical isolates of HCMV is initially focal and progresses slowly. The foci consist of a few enlarged refractile cells that contain brownish cytoplasmic granules. As infection spreads to surrounding cells, foci gradually enlarge and over a period of weeks undergo central degeneration; formation of satellite foci is frequent. When the inoculum contains unusually large quantities of virus, the initial infection may involve the entire monolayer. Under these circumstances generalized cell roundings

may be evident as early as 24 hours post-inoculation. Additional characteristics of HCMV are its ability to propagate exclusively in homologous fibroblasts and, in early passages, fresh isolates produce only cell associated virus (119).

In general, subconfluent, actively growing cells yield more infectious virus than confluent, contact-inhibited cells. Cultures of human embryonic lung (HEL) cells in different physiological states were studied for their susceptibility to infection with human cytomegalovirus with respect to production of infectious virus, synthesis of viral antigens, and virus-induced stimulation of cellular DNA synthesis (18). The higher yield of infectious virus was correlated with a greater percentage of cells producing viral antigens within the first 48 hours after infection. DNA activity in the cells is not shut off. In confluent cultures, 25 to 50% of the cells produced viral antigens within the first 48 hours post infection. However all the cells in subconfluent cultures were susceptible.

Several reasons have been proposed to explain the slow appearance of CPE with wild isolates:

1) Unadapted wild strain: with a well adapted strain of CMV, such as AD 169, according to IFA analysis, a homogeneous nuclear fluorescence appears within 1-3 hours after inoculation of HCMV in fibroblast cells (48). By 24 hours, aggregates of antigen appear in the nucleus, the cytoplasm strains diffusely, and a paranuclear inclusion is easily identified (73). Viral DNA replication at 48-72 hours after infection is accompanied by a prominent staining for the newly

formed nuclear inclusion, as well as the perinuclear cytoplasmic inclusion (73). Pseudopodic extensions of the cytoplasm are frequently observed and nuclear fluorescence in adjacent cells indicates extracellular spread of infection (73). At approximately 24 hours after infection, a receptor for the Fc portion of IgG develops in the cytoplasm (60). By contrast with the above, in primary isolation of a clinical isolate, light microscopy and EM studies revealed cellular changes similar to those described before but with several important exceptions (58). Most cytoplasmic nucleocapsids have incomplete cores and the paranuclear cytoplasmic inclusion is surrounded by lysosomes.

In addition, early cell death from lysis contributes to defective virion formation. Also lysosomal injury is responsible for the difficulty in adapting fresh isolates to cell culture. Therefore, the virus-cell interaction varies depends on whether the virus is laboratory adapted or a recent clinical isolate (73). Fresh isolates must be serially passed at low multiplicity of infection in vitro in order to adapt the strains to cell culture (73). The mechanism responsible for the change in the replicative ability is unknown.

2) Presence of defective interfering particles (DI) (This has already been described in 2.1.3.5.).

3) Human fibroblasts at a high passage level can slow down the CPE appearance for diagnostic work, the tissue of origin of

human fibroblasts is apparently of little consequence, since various tissues have been used successfully (73). However, high passage of these cell strains may have too short a lifespan to be ideal for isolation of HCMV (73).

4) Low viral titer in the inoculum: when large quantities of HCMV are present in the inoculum, generalized cell rounding may appear within 24 hours (48, 73).

5) The physiological state of human embryonic lung cells affects their response to human cytomegalovirus (18) (described in 2.3.3.): a much larger proportion of the cells in the subconfluent than in the confluent cultures synthesized viral antigens. The physiological state of the cells at the time of infection affects their ability to synthesize detectable amount of CMV antigens and to support CMV growth (18).

2.3.4. Complement fixation test (CFT)

The antigens fixing complement have already been described in 2.1.6. This test, first developed by Rowe et al (104) in 1956, was modified by Medearis (78) in 1964. Most laboratories employ the micromethod, which is accurate, requires small amounts of serum and is convenient for large-scale testing (7, 40, 67). The complement fixation test requires an advanced degree of CPE for the preparation of a good antigen (29). This test can be used for the detection of antigens or antibodies.

The CFT is based on the fixation of complement by antigen-antibody complexes. The fixation of a known amount of complement is demonstrated by testing for residual complement with an indicator system comprising sheep RBC's sensitized by specific hemolysin (antiserum to sheep RBC). The absence of hemolysis indicates fixation of complement. Because the known sera used to identify the unknown CMV antigen contains complement, it must be inactivated by heating at 56°C for 30 min. before testing (35). Human sera should be pretested for the absence of herpes simplex, varicella zoster and EB antibodies to prevent false positive results.

The fixation of complement may be allowed to take place at 37°C for a short period (1-1½ hours) or at 4°C for a long period (overnight). The cold overnight fixation increases the sensitivity without a corresponding increase in anticomplementary reactions (35).

2.3.5. Neutralization test

The antigens responsible for neutralization have already been described in 2.1.6.

The infectious virus after combination with the specific antibody, becomes neutralized, i.e. rendered non infectious. This reaction can be performed with viruses which neither hemagglutinate nor react in CFT. In principle, virus and antiserum are mixed, allowed to react for an appropriate time, and the mixture inoculated into a susceptible host system which is then observed for signs of infection. By the use of specific antisera, unknown viruses may be identified (35).

The test is complicated by the fact that cell-free viral preparations must be used and they are difficult to obtain with fresh isolates (HCMV in preliminary passage is intracellular). Also, human sera must be pretested for the absence of HS and V. Zoster antibodies. It requires controls with HS and VZ antihuman sera. Furthermore, the neutralization test requires many days to complete, especially with low titer isolates (29).

2.3.6. Indirect immunufluorescence (IFA) technique

This technique was first described by Weller and Coons in 1954 (134). It enables one to visualize microscopically antigens or deposits of antibodies and/or complement on tissues or cells and detect (73):

a) Cell surface and intracellular antigenic constituents and determine their location within the tissue.

b) The location and type of antibody and complement deposited in the tissue.

c) Virus particles within the tissue.

d) Circulating antibodies to pathogenic bacteria, viruses, fungi and protozoa.

The basis of this technique (73) lies in the fact that ultraviolet irradiation is converted to visible light after it strikes a fluorescent compound. The latter extracts energy from the invisible short wavelength (ultraviolet, U-V) irradiation transforming it to visible light of longer wavelength and lesser energy. Thus, if visible light emanating from U-V source is

blocked and only U-V irradiation is permitted to strike the tissue-fixed fluorescein-conjugated compound, visible light will be emitted from the tissue specimen wherever the fluoresceinated compound has localized. This test is useful for CMV diagnosis because the identification in cell culture is time consuming.

The principle of indirect immunofluorescent assay is illustrated in Figure 3 (22).

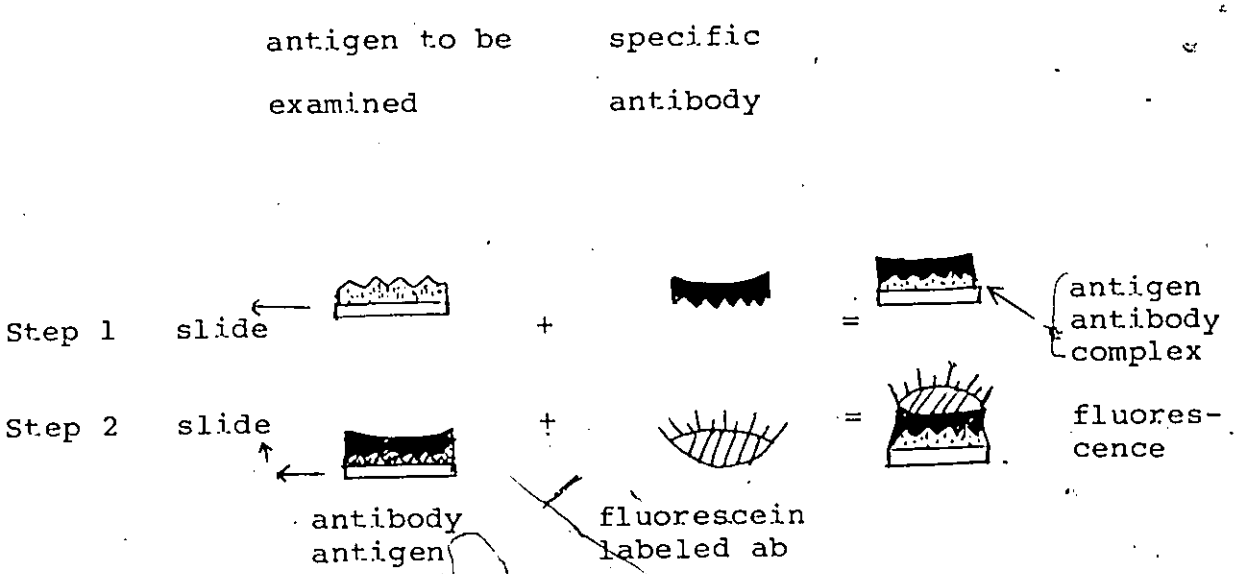


FIGURE 3: Principle of indirect immunofluorescent assay.

2.3.7. Anti-complement immunofluorescence

The anti-complement immunofluorescence (ACIF) test is based upon the binding of complement to viral antigen-antibody complexes in CMV infected cells and the detection of the bound complement with fluorescein labeled antibodies to the C_3 component by using a UV microscope (108).

Specific identification was not attempted by ACIF until isolates showed a clear out CPE resembling that of CMV (108). However, it was indicated that ACIF can be used to detect CMV antigen in infected cells before CPE is apparent (28) or detect CMV antigen directly in infected tissues (53).

Selection of proper immune sera is an important factor in this assay (54).

2.3.8. Rapid detection and quantitation of human CMV in urine by DNA-DNA hybridization

Recent advances in recombinant DNA methods have resulted in the availability of relatively abundant supplies of cloned DNA fragments from which DNA hybridization probes may be easily prepared (12). After immobilization on nitrocellulose paper, denatured microbial DNA may be specifically detected by hybridization with radioactively labeled probes prepared from DNA fragments of the organism of interest. Chou and Merigan in 1983 (12) detected CMV DNA in clinical urine specimens.

The following points should be noted in such studies:

- The efficiency with which viral DNA in a urine sample is immobilized on nitrocellulose paper depends in part on the

completeness of sedimentation of viral particles during ultracentrifugation and the affinity of DNA for nitrocellulose in the presence of extraneous substances in urine. These factors influence the intensity of the hybridization signal. The sensitivity is only equivalent to $10^3 TCID_{50}$.

- The DNA hybridization signal as far as quantitation of infective virus is concerned would also be influenced by the numerous defective non infectious particles that are produced in the course of CMV replication.

- Although the use of P32 labelled DNA for this hybridization assay is feasible in the research setting, widespread application of this technology in clinical practice will require the development of more convenient labeling and detection systems for probe DNA, such as those based on enzymatic reactions. Such systems, e.g. biotin-avidin, are under development in several centers (100).

3. METHODOLOGY AND MATERIALS

3.1. Cell cultures

3.2. Media used

3.3. Trypsin and trypsinization method

3.4. Freezing medium and freezing of cells

3.5. Specimens studied

3.6. Identification of CMV by immunofluorescent assay

3.7. Complement fixation test

3.1. Cell cultures

3.1.1. Human fetal lung fibroblasts (HFL)

The diploid semi-continuous cell lines used in this study were developed by Mr. B. McCulloch at the Children's Hospital of Eastern Ontario. These cells were kept in liquid nitrogen (-196°C) at a concentration of 10×10^6 cells/ml. They were used at pass level 11 to 13. They have been shown to be just as sensitive as MRC_5 for CMV isolation (Phipps and McCulloch, unpublished data).

The preparation of human embryonic fibroblasts for routine work was as follows:

The frozen cells were thawed and resuspended in growth medium (described in 3.2.2.) seeded in a tissue culture flask (75 cm^2) and incubated at 37°C for 48 hours till the cells showed confluency. These cells were trypsinized, counted and resuspended in maintenance medium (described in 3.2.1.) at a concentration of 1×10^5 cells/ml in 100 x 13 mm tubes. The tubes were stoppered and incubated at 37°C stationary in racks at an angle of about 5°. In this position, the cells settled as a monolayer of about 2.5 cm length on the lower surface of the tube. These cells could be observed for 2 weeks for the isolation of CMV. They were then passed blindly to fresh cells and observed for another period of 2 weeks.

3.1.2. Green monkey kidney (primary) (GMK)

These were used to show that the virus isolated was neither herpes simplex nor varicella zoster. It is known that HCMV will not grow in GMK while H.S.V. and V.Z.V. can.

The GMK cells were received as a suspension from Flow laboratories at a concentration of 1×10^6 cells/ml. They were diluted to 2×10^5 cells/ml.

3.2. Media used

3.2.1. Maintenance medium

It is composed of 2% fetal calf serum (FCS) in Eagle's MEM (TABLE IV).

Antibiotics are added to the maintenance medium at a concentration of:

penicillin:	100 IU/ml
gentamycin:	25 mcg/ml
fungizone:	1 mcg/ml

3.2.2. Growth medium

It is composed of 10% fetal calf serum in Eagle's medium (TABLE IV).

The Eagle's medium is supplied by the Grand Island Biological Company.

3.2.3. Specimen inoculation medium (components in TABLE V)

Preparation: to one litre of Hank's Salts add:

- 2 g Bovine Serum Albumin Power (Fraction V)
- 90 ml of 0.280 M HEPES in H_2O (6.67% solution)
- 40 ml of stock fungizone solution (0.25 mg/ml)
- 40 ml of stock penicillin/gentamycin solution:

Total volume = 1170 ml.

3.3 Trypsin and Trypsinization method

For the most efficient dissociation of cells, enzyme solutions and chelating agents should be prepared in saline solutions free from calcium and magnesium, as the presence of these ions increases the stability of the intercellular matrix (35).

Trypsin in particular, has been used extensively for disaggregating tissue (93).

The trypsin in particular, has been used extensively for disaggregating tissue (93).

The trypsin used in the research was supplied by Flow Laboratories in the form of 0.5% trypsin/0.02% EDTA.

EDTA (93): Certain tissues, especially epithelial tissues, seem to require divalent cations, particularly calcium and magnesium, for their integrity. If these ions are removed by substances which bind them (chelating agents), the tissues disrupt more easily. Chelating agents most commonly used are citrate and ethylene-diamine-tetra-acetic acid (EDTA, versene or sequestrene).

Trypsinization method:

- The old medium is removed from the cell culture.
- The monolayer is washed with 1 ml of warmed trypsin to remove any extra calcium and magnesium; another 1 ml of trypsin is added and left in contact with the cells for 15-30 sec. at 37°C, until microscopic examination shows that the cells are completely dislodged into the medium. The cells are

resuspended in some of the old medium and passed to new fresh cells for subculturing, preparing slides or freezing.

TABLE IV: Components of growth and maintenance medium

	Component	Maintenance Medium Dulbecco's Modification of Eagles Medium	Growth Medium Eagle's MEM with Earle's Salts with NEAA*
		mg/L	mg/L
Inorganic Salts	$CaCl_2$ (anhyd.)	200.00	200.00
	$Fe(NO_3)$ $9H_2O$	0.10	
	$NaHCO_3$	3700.00	2200.00
	KCl	400.00	400.00
	$MgSO_4$ (anhyd.)	97.67	97.67
	NaCl	6400.00	6800.00
	NaH_2PO_4 H_2O	125.00	140.00
Other Components	Glucose	4500.00	2000.00
	Phenol red	15.00	10.00
Amino Acids	L-Alanine		8.90
	L-Arginine. HCl	84.00	126.00
	L-Asparagine. H_2O		15.00
	L-Aspartic acid		13.30
	L-Cystine. 2 HCl	62.57	31.29
	L-Glutamic acid		14.70
	L-Glutamine	584.00	292.00
	Glycine	30.00	7.50
	L-Histidine HCl. H_2O	42.00	42.00
	L-isoleucine	105.00	52.00
	L-Leucine	105.00	52.00
	L-Lysine HCl	146.00	72.50
	L-Methionine	30.00	15.00
	L-Phenylalanine	66.00	32.00
	L-Proline		11.50
	L-Serine	42.00	10.50
	L-Threonine	95.00	48.00
	L-Tryptophan	16.00	10.00
	L-Tyrosine (Disodium salt)	104.20	52.10
	L-Valine	94.00	46.00
Vitamins	D-Ca pantothenate	4.00	1.00
	Choline Chloride	4.00	1.00
	Folic acid	4.00	1.00
	i-Inositol	7.20	2.00
	Ninotinamide	4.00	1.00
	Pyridoxal HCl	4.00	1.00
	Riboflavin	0.40	.10
	Thiamine HCl	4.00	1.00

*NEAA = Non-Essential Amino Acids
MEM = Minimal Essential Medium

Component	mg/L
$Ca\ Cl_2$	120.0
K Cl	342.0
$KH_2\ PO_4$	51.3
$Mg\ SO_4$	83.5
Na Cl	6838.0
$Na_2\ HPO_4$	40.0
$Na\ HCO_3$	300.0
Glucose	855.0
Phenol Red (phenolsulfonphthalein)	14.5
BSA (Bovine Serum Albumin)	1710.0
HEPES (N'-2-Hydroxyethylpiperazine - N' - Ethanesulfonic acid)	5128.0
Fungizone (Amphotericin B)	8.6
Gentamycin Sulphate	213.7
Penicillin G Sodium: 8.55×10^5 IU/L	

TABLE V: Components of Specimen Inoculation Medium

3.4. Freezing medium and freezing of cells

The use of glycerol in the freezing medium enables the long-term preservation (from days to years) of the cells. The glycerol suppresses the tendency to form intracellular ice so increasing the potential for larger number of cells to survive (59, 110).

The cells were kept for 24 hours at -80°C and then transferred to the -196°C liquid nitrogen freezer.

The freezing medium consisted of a mixture of:

5 ml of diethylene glycerol (Fisher)
10 ml of glycerine (Fisher)
85 ml of growth medium (no antibiotics)

100 ml of freezing medium

The mixture was stirred using a magnetic stirrer for 5 - 10 min. and then filtered using Nalgene sterilization filter unit type S, pore size 0.20 micron. The freezing medium was kept at 4°C and used within 2 weeks.

Method for freezing cells:

- The cells were trypsinized as described in 3.3.
- The trypsinized cells were resuspended in 0.25 ml of freezing medium.
- The mixture was transferred to a freezing ampoule which was sealed and kept at -80°C freezer and then transferred to the (-196°C) freezer 24 hours later for long term storage.

3.5. Specimens studied

3.5.1. Urine specimens

Urines were collected at the Children's Hospital of Eastern Ontario (CHEO) and at the Ottawa General Hospital (OGH). The specimens were grouped according to the age into the following groups:

Group	A	0 - 6 months
"	B	7 - 12 "
"	C	1 - 4 yrs
"	D	5 - 9 "
"	E	10 - 14 "
"	Fc	15 - 22 "
"	Fg	15 - 24 "
"	G	25 - 34 "
"	H	35 - 44 "
"	I	45 - 54 "
"	J	// 55 "
"	P	pregnant women in the first trimester of pregnancy

Urines from groups A, B, C, D, E and Fc were collected from inpatients and outpatients at the CHEO.

Urines from groups Fg, G, H, I, J and P were collected from outpatients of the OGH.

3.5.1.1. Preparation of urines

For reasons of economy, within each age group, 5 urines were pooled together (2 ml each) and manipulated as one specimen.

Each pool was neutralized with 0.1 N NaoH in the presence of phenol red indicator.

The neutralized urine was centrifuged at 1400 g for 15 min at 4°C. Previous work in the laboratory showed that urines centrifuged at 1400 g showed very few contamination in culture. However, if not centrifuged before being inoculated into cell culture, the rate of contamination was at least 50%. It was argued therefore that there should be a far greater concentration of bacteria in the sediment than in the supernatant fluid. For this reason, the concentration of antibiotics used in this study when deposits were cultured was triple the concentration used when supernatant fluids were cultured.

The supernatant and the sediments were separated and antibiotics were added in the following concentrations.

Penicillin — supernatant 2500 IU/ml of supernatant
sediment 7500 IU/sed. from 10 ml of urines

Gentamycin — supernatant 625 mcg/ml of supernatant
sediment 1875 mcg/sed. from 10 ml of urines

Fungizone — supernatant 25 mcg/ml of supernatant
sediment 75 mcg/sed. from 10 ml of urines

The mixture of urine and antibiotics was left at room temperature for one hour before inoculation.

3.5.1.2. Inoculation of urines into cultures

Two drops each of the supernatant and sediment were inoculated into 2 tubes of HFL: one for the supernatant and one for the sediment.

The cultures were observed twice a week for cytopathic effect (CPE) and toxicity.

In the presence of cytopathic effect (CPE), the cultures were observed three times a week for the progression of CPE, then passed into a 25 cm^2 30 ml flask. When CPE was well developed, the cells were trypsinized and frozen as described in 3.4.

In case of toxicity, the tubes were trypsinized and passed into freshly prepared HFL cells.

All the cultures were kept for 5 - 6 weeks and blindly passed after 2 weeks to new HFL cells.

All positive cultures were passed to GMK (described in 3.1.2.).

3.5.2. Cervical swabs

Cervical swabs were collected from normal healthy pregnant women seen routinely at a private obstetrical practice in the third trimester (28 - 40 weeks) of pregnancy and transferred to tubes containing specimen inoculation medium (described in 3.2.3.).

The swabs were left in the diluent for approximately 4 hours. Then three drops of each specimen were inoculated to 2

HFL tubes which were processed as described for the urines specimens (3.5.4.).

3.5.3. Sera studied by the complement fixation test

Sera sent routinely to the Regional Virus Laboratory over the period of six months from January to June 1982 were analysed by complement fixation test using a CMV CF antigen prepared by glycine extraction and obtained from the Laboratory Center for Disease Control (LCDC). Sera were initially tested at 1/20 using the method described under 3.7., and the age groups described under 3.5.1. were studied with the exclusion of the pregnant women group. The data obtained were compared with the results of the CMV isolation study.

3.6. Identification of CMV by Immunofluorescent assays

3.6.1. Preparation of IFA slides

- Cell culture showing CPE were trypsinized and resuspended in 1 ml of medium
- 8 wells IFA slides which had been cleaned with 7 X detergent were used
- One small drop of cell suspension was placed on each well
- The slide was placed in a petri dish and left for 5 min
- The medium from each well was drawn off using a piece of torn bibulous paper
- The slide was covered and allowed to dry, and then fixed in acetone for 20 min
- The slides were kept at -80°C.

3.6.2. Reagents used in the IFA tests

1. CMV monoclonal antibodies (66) early and late (ascitic fluid) were obtained from Biotech. Laboratories, Betheda, Maryland (133). The early antibody was directed against an early nuclear antigen and the late antibody directed against a late nuclear antigen. Neither antibody neutralized CMV infectivity. These antibodies were diluted in IFA buffer and kept at 4°C. Their optimal dilution was 1:10, as determined by preliminary checkerboard titrations.

2. Conjugate: An FITC goat antimouse was obtained from Microbiological Research Corp. The Fab fragment was conjugated with fluorescin isothiocyanate. For use it was diluted in the presence of 0.025% Evans's blue as a counterstain. The optimal dilution was 1:10, as determined by checkerboard titrations.

3. Phosphate buffered saline (PBS) pH 7.4 2 was used as diluent.

4. Buffered glycerol pH 8.0 was used to mount the IF slide.

5. CMV antigen positive slides from Electro-Nucleonics were used as positive controls and kept at +4°C. Each well contained a mixture of fixed infected and uninfected cells.

6. Negative control slides consisted of uninfected human fetal lung cells, cells infected with herpes simplex type 1 and 2, V-Z and EB, the latter obtained from Electro-Nucleonics.

7. A Leitz Laborlux 11 incident UV microscope was used. It was fitted with a superpressure mercury vapor lamp HBO 50 W and an incident filter block T_2 for FITC work.

8. Photographs were taken with a Reichert Photo-automatic camera, using Kodacolor film 1000 ASA at a magnification of 438X.

3.6.3. The IFA technique

1. The required slides were removed from -80°C freezer, including the positive and negative control slides, and allowed to warm up.

2. Each well was covered with approximately 10 ml of the desired monoclonal antibody.

3. The slides were placed in a covered, moist chamber and incubated at 37°C for 30 min.

4. The slides were rinsed briefly in a light stream of buffer which was not directed into the wells, and then rinsed thoroughly in a staining dish of buffer for 10 min. The buffer was changed twice during the 10 min rinse. The buffer was agitated on a magnetic stirrer.

5. Slides were removed from the dish and air dried.

6. Each well was again covered with approximately 10 ml per well of the desired dilution of the conjugate.

7. Steps 5 and 6 were repeated.

8. One small drop of buffered glycerol was placed on each well and the slides were covered with a 22 x 50 mm, No. 1 thickness coverslip.

9. The slides were examined at a magnification of 438X. For best reading, the slides were examined immediately. The slides were kept cool (2-8°C), stored in the dark and sealed or kept humid to prevent the mounting fluid from drying.

3.7. Complement fixation test

A four-volume micro-test in disposable plastic plates was employed (124) using 0.025 ml each of antigen, antiserum, complement and 1% sensitized RBC. Standardization of complement, haemolysin, antigens and standard antisera is necessary before carrying out the test.

3.7.1. Materials and equipment

The first four items listed below, namely buffer, sheep blood, haemolysin and complement, must be kept at 4°C when not in use.

- "Veronal" buffer (VB). This was used as diluent throughout the test. It contains calcium and magnesium which are necessary for full fixation of complement at high dilutions of serum. VB containing 0.08 percent sodium azide (AVB) was used for dilutions of antigens as these must be kept for several days. VB containing 0.1 percent bovine albumin (VBA) was used to stabilize complement when testing in the absence of other protein such as antigen or antiserum.
- Sheep blood: This was obtained weekly from Flow Laboratories but was washed and standardized every day. A 1 percent RBC suspension standardized volumetrically was made on the day of the test as follows. The cells were washed in VB until the supernatant was clear. For volumetric standardization a sample of the cell suspension was centrifuged in two hematocrit tubes in a horizontal bench centrifuge at 3000 rev/min for 20 min. The diluent to make a 1 percent suspension was calculated from the packed volume of cells.
- Hemolysin: (horse or rabbit hemolytic serum, glycerinated, for sheep RBC). A stock dilution of 1/10 in AVB was prepared each week from which daily working dilutions were prepared.
- Complement: (freeze-dried preparation of preserved guinea-pig serum. The complement when stored at -80°C will retain its activity unchanged without preservatives for one year. Thus when stored in this manner it could be titrated very precisely to determine that concentration giving five 50% hemolytic units. A 1/10 dilution of fresh guinea-pig serum in saline was prepared; further dilution was made with VB to the strength

required. After dilution, complement is unstable. It should therefore be prepared immediately before use and kept on melting ice until distributed to the plates.

- Viral antigens: The CMV antigen was produced in MRC_5 cells and obtained from the Laboratory Center for Disease Control (LCDC).
- Uninfected control antigen: This was made from similar uninfected MRC_5 cell cultures.
- Positive control antisera: Human convalescent sera were used, preferably pooled to minimize variation in antigen requirements between individual sera.
- Microtechnique equipment (total test volume 0.1 ml): A hand multidiluter handle for twelve diluters each holding 0.025 ml for preparing doubling dilutions of sera was used; for large-scale tests an automated microdiluter may be used. Single channel reagent dispensers - for distributing 0.025 ml volumes of diluent, complement and sensitized sheep cells were used.

3.7.2. Standardization of complement and haemolytic serum

The haemolytic titer of each new batch of complement must be determined by chessboard titration against each new batch of lysin (SRBCs antisera). For this purpose doubling serial dilutions were satisfactory for haemolysin and for complement the difference between dilutions should be less.

Procedure

1. Prepare serial dilutions of complement (Table VI) with a 20 percent difference in concentration between each, as follows: place eight containers in a rack (1 Universal, 7 Bijoux). With automatic pipette put 1 ml VB in each Bijou. In a Universal container, make a 1/60 dilution of complement (0.3 ml complement, 2.1 ml deionized water, 12 ml VB); mix thoroughly but gently avoiding bubles, and pass 4 ml (4 x 1 ml) with an automatic pipette from container 1 to 2; mix and repeat passing 4 ml from container 2 to 3 and so on to container 8. Store on melting ice until required.

2. With a pipette dropper add 2 volumes of 0.025 ml VB containing 0.1 percent bovine albumin to each well (to replace 1 volume serum and 1 volume antigen in the test proper); put an additional 1 volume (to replace complement) in each haemolytic serum control well.

3. Pipette 0.025 ml complement dilutions in each well of the corresponding column starting with the highest dilution.

4. Wrap plate securely in foil to prevent evaporation and stand at 4°C overnight.

5. The next morning, prepare in 1 ml amounts a series of doubling dilutions of haemolysin from 1/50 to 1/800 and one tube containing VB only as control. Mix each dilution with an

equal volume of 1 per cent washed sheep RBC, and sensitize for 10 min in water bath at 37°C or at RT for at least 30 min.

6. Remove plates from refrigerator and warm on 37°C hot-plate for 20 min.

7. Place 0.025 ml volumes of sensitized cells into wells starting with the control cells without haemolysin and continuing with the highest dilution of haemolysin. Seal with tape, shake and place in incubator room on hot-plate for 40 min at 37°C, shaking on a microshaker after about 15 min, 30 min, and again on removal from incubator.

8. Place Plates in refrigerator overnight or for several hours at room temperature until cells have settled, then read.

The optimal sensitizing dose (OSD) of haemolytic serum is the dilution giving most lysis with the highest dilution of complement, i.e. 1/400 in Table VI. Since 50 per cent haemolysis is a more sensitive and reproducible index of haemolytic activity of complement than 100 percent lysis, one unit of complement (HD_{50}) is the dilution giving 50 percent lysis with the OSD of haemolysin, e.g. 1/184 in Table VI. In the test proper, complement is used at $4HD_{50}$ units, i.e. 1/46 in our example.

Complement Fixation Tests

Reciprocal dilutions of haemolytic serum in 1 per cent sensitized cells	Reciprocal dilutions of complement								Haemolytic serum controls
	60	75	94	118	148	184	230	288	
100	tr	tr	1	2	2	3	4	4	4
200	0	0	tr	1	2	3	4	4	4
400	0	0	0	tr	1	2	3	4	4
800	0	0	tr	1	2	3	4	4	4
1600	tr	1	2	3	4	4	4	4	4
Complement controls	4	4	4	4	4	4	4	4	

0 = no cells remaining
tr = approximately 10 per cent cells remaining
1 = approximately 25 per cent cells remaining
2 = approximately 50 per cent cells remaining
3 = approximately 75 per cent cells remaining
4 = approximately 100 per cent cells remaining

TABLE VI: Titration of complement and haemolytic serum

3.7.3. Standardization of antigen and antisera

It is necessary to obtain the optimal dilution of each antigen and the titer of the corresponding standard antiserum. Controls for nonspecific reactivity must be included to confirm the specificity of the antigen-antibody reaction.

1. Using 1 ml automatic pipette prepare in tubes serial doubling dilutions of (a) antigen and (b) antiserum (inactivated at 56°C for 30 min); if antigen and antiserum have not been previously tested dilutions should range from 1/2 for antigen and from 1/8 for antiserum; otherwise, from four times previous optimal dilution and titer respectively.

2. Carry out two-dimensional (chessboard) titration of antigen and antiserum (Table VI) using separate droppers for each reagent. Include the following controls: antigen dilutions against both $4HD_{50}$ and $2HD_{50}$ complement (VB replacing serum), antiserum dilutions also against 4 and $2HD_{50}$ complement (VB replacing antigen), nonspecific reactivity (testing each dilution of antiserum against an uninfected control antigen at concentrations equal to the two highest concentrations of specific antigen):

   a) put 1 volume (0.025 ml) VB into control wells (antiserum columns and antigen now controls).

   b) put respectively 2, 2, 2, 2 and 3 volumes VBA in five wells - for complement controls at 4, 2, 1, 1/2 and 0 (cell control) HD_{50}.

   c) Add 1 volume of antiserum dilutions in rows as indicated, starting from highest dilution.

   d) add 1 volume complement ($4HD_{50}$) to each well; for complement, antigen and antiserum controls add indicated dose.

   e) add 1 volume of antigen dilutions in columns starting from highest dilution.

   Then proceed as for steps 10-14 in Test proper below.

Most antigen-antibody patterns fall into two main groups as exemplified in Table VII (a) giving a peak optimal antigen dilution, or (b) giving a plateau of optimal antigen dilution. An antigen showing a peak pattern should be used at the optimal

TABLE VII: Standardization of antigens and antisera

(a) Showing peak optimal antigen titer

Antiserum dilutions	Antigen dilutions							Uninfected control antigen dilutions		Antiserum controls		Complement controls
	2	4	8	16	32	64	128	2	4	$4HD_{50}$	$2HD_{50}$	(HD_{50})
8	4	4	4	4	3	0	0	tr	0	0	tr	0 (4)
16	4	4	4	4	2	0	0	0	0	0	0	0 (2)
32	2	3	[4]	3	1	0	0	0	0	0	0	1 (1)
64	0	1	2	tr	0	0	0	0	0	0	0	2 (1/2)
128	0	0	0	0	0	0	0	0	0	0	0	4 (0)
256	0	0	0	0	0	0	0	0	0	0	0	
Antigen $4HD_{50}$	0	0	0	0	0	0	0	0	0			
controls $2HD_{50}$	0	0	0	0	0	0	0	0	0			

(b) Showing plateau antigen titer

Antiserum dilutions	Antigen dilutions							Uninfected control antigen dilutions		Antiserum controls		Complement controls
	2	4	8	16	32	64	128	2	4	$4HD_{50}$	$2HD_{50}$	(HD_{50})
8	4	4	4	4	3	tr	0	0	0	0	tr	0 (4)
16	4	4	4	4	2	0	0	0	0	0	0	0 (2)
32	4	4	4	[4]	1	0	0	0	0	0	0	1 (1)
64	2	2	2	2	0	0	0	0	0	0	0	2 (1/2)
128	0	0	0	0	0	0	0	0	0	0	0	4 (0)
256	0	0	0	0	0	0	0	0	0	0	0	
Antigen $4HD_{50}$	0	0	0	0	0	0	0	0	0			
controls $2HD_{50}$	2	1	0	0	0	0	0	0	0			

All dilutions expressed as reciprocals

peak dilution (OPD). With an antigen showing a plateau pattern 1 unit of antigen may be taken as the highest dilution of antigen giving complete fixation with the highest peak dilution (OPD). With an antigen showing a plateau pattern 1 unit of antigen may be taken as the highest dilution of antigen giving complete fixation with the highest dilution of serum; the antigen should be used at 2 or 4 units (preferably) 4 if not anticomplementary. A plateau pattern often indicates anti-complementary activity at low antigen dilutions sometimes detected with $2HD_{50}$ but not with $4HD_{50}$ (Table VII b).

The titer of the serum is the highest dilution of serum which gives a reading of 4 or 3 with the antigen. Complete fixation endpoints are marked ([ ]) in Table VII a and VII b. The antigens in both samples would be used at 1/8, i.e. OPD in (a); twice endpoint in (b). The titer of each serum is 1/32.

3.7.4. Serological Tests

Doubling dilutions of sera are made with a multi-micro-diluter and tested in parallel against relevant antigens: the reagents, in order of addition, comprise 1 volume (0.025 ml) each of serum dilution, complement $4HD_{50}$, antigen at OPD or at 2-4 units, and 1 per cent sensitized sheep erythrocytes. Controls comprise (a) anticomplementary activity: serum 1/8 with no antigen tested against 4 and $2HD_{50}$ complement; (b) complement (with 2 volumes VBA replacing antigen and antiserum): HD_{50} units of 4, 2, 1, 1/2 and 0 (cell control); (c) specific reagent control: standard positive antiserum titrated from 4 times titer

(4T) to 1/4 titer (T/4) against the dilution of specific antigen used in test; controls of specific antigen (no antiserum) plus 4 and $2HD_{50}$ complement and standard antiserum at lowest dilution (4T) without antigen plus 4 and $2HD_{50}$ complement are also included. Uninfected control antigen can also be used to test for nonspecific reactivity of serum, but this is not routinely required.

Procedure

1. Heat 1/4 serum dilutions (0.2 ml serum, 0.6 ml AVB) at 56°C for 30 min to inactivate complement.

2. With reagent dispenser put one volume VB as indicated (Table VIII) into wells 2-12 for each test serum and for each specific reagent control (when several antigens are used it is more convenient to put specific reagent controls together on a separate plate).

3. With pipette dropper add VBA to 5 complement control wells: 2, 2, 2, 2 and 3 volumes respectively for HD_{50} complement at 4, 2, 1, 1/2 and 0 (cell control).

4. Using automatic pipette, with separate pipette and for each serum, place approximately 0.1 ml of each test serum into the master well (number 1).

5. Make doubling dilutions (0.025 ml) by transferring the diluters from wells 1-2 and so on to 8; mix well at each transfer by rotating the diluters 4 times. Blot diluters dry. Make three further transfers of sera from wells 1 for (a) non-specific reactivity (with doubling dilutions wells 9-10) and (b) serum controls (1/8 dilutions in wells 11 and 12). Blot diluters between (a) and (b). Clean diluters.

6. Make doubling dilutions of standard sera from 4T to T/4: include serum controls (4T in wells 11 and 12).

7. With reagent dispenser add 1 volume complement $4HD_{50}$ to wells 2-11 in test rows, and to wells 2-7, 9 and 11 in control rows. Add 1 volume complement $2HD_{50}$ to well 12 of test rows and wells 8, 10 and 12 of control rows; for complement controls add 1 volume 4, 2, 1 and 1/2 HD_{50} respectively as indicated.

8. With pipette dropper add 1 volume of antigen at OPD or at 2-4 units to test serum dilutions (wells 2-8) and then to specific reagent row (wells 2-8).

9. With a fresh pipette dropper add appropriate uninfected control antigen (same dilution as true antigen) to wells 9-10, and to uninfected control antigen control (wells 9-10) of specific reagent control.

TABLE VIII: Procedures for CF tests on sera

(a) Serum dilutions in microplate for CF test (one row of microtiter plate is shown)

	Master	With OPD antigen							With uninfected control antigen		Serum control (no antigen) VBS	
Well No.	1	2	3	4	5	6	7	8	9	10	11	12
Test serum dilutions	4	8	16	32	64	128	256	512	8	16	8 $4HD_{50}$	8 $2HD_{50}$

(b) Reagents controls

	Master	With OPD antigen					Antigen Control (OPD)		Uninfected antigen control		Serum control (no antigen) VBS	
Well No.	1	2	3	4	5	6	7	8	9	10	11	12
Specific antiserum	8X titre 8T	4T	2T	T	T/2	T/4	No antiserum (VBS) $4HD_{50}$	No antiserum (VBS) $2HD_{50}$	No antiserum (VBS) $4HD_{50}$	No antiserum (VBS) $2HD_{50}$	4T $4HD_{50}$	4T $2HD_{50}$
	Complement Control											
	Complement HD_{50} 4 2 1 1/2 0 (cell control)											

10. With fresh dropper add 1 volume VBS to wells 11 and 12 to make up 3 volumes.

11. Stack plates, wrap securely with foil and stand overnight at 4°C.

12. Next morning sensitize washed 1 per cent sheep RBC in a conical flask by slow addition with thorough mixing of an equal volume of haemolysin (final mixture, 5 per cent cells with optimal sensitizing dilution of haemolysin). Always add lysin to cells as addition of cells to lysin may result in uneven sensitization. Incubate in water bath at 37°C for 10 min or at room temperature for at least 30 min.

13. Remove plates from refrigerator and warm for 20 min at 37°C. Add 0.025 ml of .5 per cent sensitized sheep cells to each well. Cover plates with sealing tape.

14. Reincubate for 40 min to allow lysis, shaking at 15, 30 and 40 min to resuspend cells using a microshaker.

15. Place plates in refrigerator overnight or for several hours at room temperature until the cells have settled.

16. Reading: The highest dilutions of serum giving a reading of 4 or 3 is taken as its titer.

4. RESULTS

4.1. Isolation findings

4.2. Serological findings

4.1. Isolation findings

Our research started by the collection of urines received at the Department of Laboratory Medicine at the CHEO for age-groups 0 to 24 years, followed by the collection of urines at the Microbiology Department of the Ottawa General Hospital for age groups 16 to 55 years. These urines were handled as described before (3.5.1.). A total number of 273 pools, representing 1305 urines were examined in cell culture. 19 (6.95%) of the pools were positive and 254 (93%) were negative for CMV (Table IX a). All the positive urines were in the age-groups 0 to 9 years with the highest proportion of the positive pools (32%) in the 1-4 years age-group.

It can be seen that this percentage rose from 1.6% in the 0 to 6 month age-group, reached a peak of 6.45% in the 1-4 age-group and became nil from age 10 onwards. Similar negative results were obtained in 65 urines collected from pregnant women in the first trimester of pregnancy. Overall, out of 1305 urines, 1.45% of them were positive for CMV. It can also be seen that the time taken for the appearance of the cytopathic effect (CPE) of CMV was rather long, ranging from 6 to 36 days, with a mean of 22 days.

In view of this low isolation rate in the urine supernatants, two other types of specimens were studies: urinary sediments and cervical swabs. Urinary sediments from one hundred pools were centrifuged and handled as described under 3.5.1., inoculated into cell culture and handled in the same way as the supernatants. These findings are presented in Table IX b. No

isolate was made from those 100 urinary sediments, in spite of the fact that some of the corresponding supernatants did yield positive results.

One hundred cervical swabs of healthy unselected pregnant women in the last trimester of pregnancy were examined as described in 3.5.2. None of these swabs were positive for CMV (Table IX c).

Group tested	Number of pools	Number of urines	Number of +ve pools (% positive)	Time taken for appearance of CPE	% of positive urines for CMV
0-6m (A)	25	125	2 (8%)	1) 11 2) 20	1.6
6-12m (b)	25	125	5 (20%)	3) 20 4) 6 5) 25 6) 11 7) 28	4
1-4y (C)	31	155	10 (32%)	8) 19 9) 24 10) 32 11) 32 12) 36 13) 26 14) 26 15) 27 16) 15 17) 15	6.45
5-9y (D)	34	170	2 (5.9%)	18) 26 19) 26	1.2
10-14y	35	175	0	-	0
15-24y	48	230	0	-	0
25-34y	15	65	0	-	0
35-44y	15	65	0	-	0
45-54y	15	65	0	-	0
55y	15	65	0	-	0
pregnant women in the first trimester of pregnancy	15	65	0	-	0
TOTAL	273	1305	19 (6.95%)		1.45

TABLE IX-a: CMV strains isolated from urine supernatants

Number of pools	Number of urines	Number of positive pools (% positive)	% of positive urines for CMV
100	500	0 (0%)	0

TABLE IX-b: Absence of CMV in urinary sediments.

Number of cervical swabs	Number of +ve	% positive
100	0	0

TABLE IX-c: Absence of CMV in cervical swabs in late pregnancy.

The 95% confidence limits of the overall isolation results are presented in table X a and X b and Figure 4. Table X b summarizes the results in three large age groups. It can be seen from the confidence limits that the excretion of CMV may in part extend beyond the 5-9 years age-group and it may be that if we looked to thousands of urines we would have found more excreters.

Age	No. of Urines	Isolates	% Positive	95% confidence limits
0-6mos	125	2	2	(0.4-6.2)
7-11mos	125	5	4	(1.4-9.1)
1-4y	155	10	6.4	(3.4-12.7)
5-9y	170	2	1	(0.1-3.9)
10-14y	175	0	0	(0.0-2.1)
15-24y	230	0	0	(0.0-1.6)
25-34y	65	0	0	(0.0-5.5)
35-44y	65	0	0	(0.0-5.5)
45-54y	65	0	0	(0.0-5.5)
55y	65	0	0	(0.0-5.5)

TABLE X-a: Confidence limits of the CMV isolation rate in different age-groups

Age	No. of Urines	Isolates	% Positive	95% confidence limits
0-11mos	250	7	3	(4.2-10.8)
1-4y	155	10	6.4	(3.4-12.7)
5-80y	835	2	1	(0.0-0.4)

TABLE X-b: Confidence limits of CMV isolation rate in three large age-groups

To identify our CMV strains, indirect immunofluorescent tests were carried out as described in 3.6.

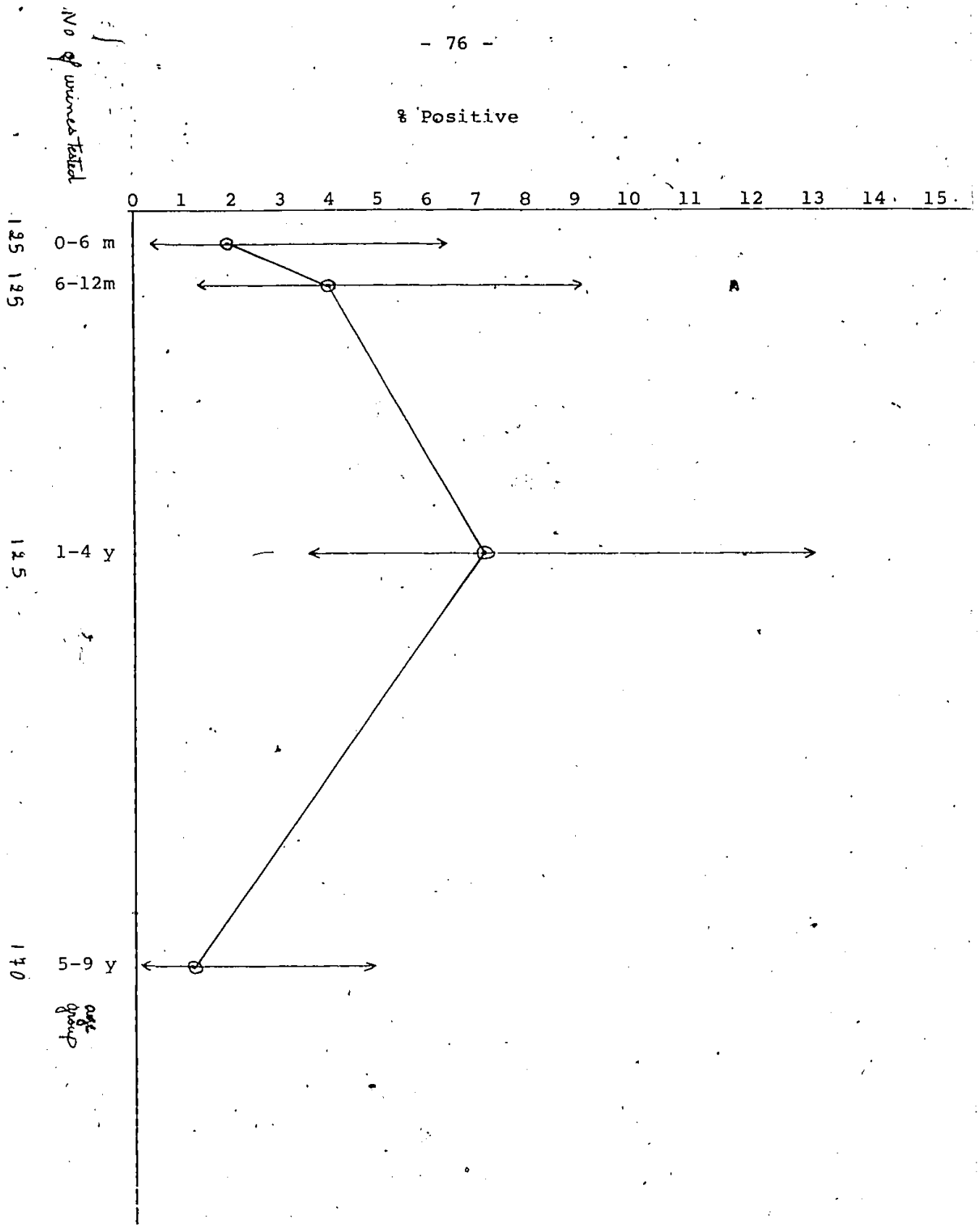
All the isolates produced specific fluorescence with the monoclonal antibodies used and only representative photomicrographs have been included.

Figures 5 and 6 show the typical nuclear and cytoplasmic fluorescence obtained with the reference CMV strain AD/169, when tested against the early and late nuclear proteins monoclonal antibodies (Biotech) and commercially available slides coated with CMV infected human fibroblasts. It can be seen that the fluorescence produced in the presence of the early nuclear protein monoclone was strictly nuclear, while that produced by the late nuclear protein was both nuclear and cytoplasmic.

The same monoclones produced no fluorescence when tested with uninfected human fibroblastic cells (figure 7), with HSV1, and HSV2 infected MRC5 (Figures 8 and 9) varicella-zoster (not shown) and Ebstein-Barr infected, lymphoblastoid cells (Figures 10,11 and 12).

All wild CMV isolates reacted specifically with the early and late monoclonal antibodies. Representative microphotographs are shown in Figure 13-17. It should be noted that the slides were made from trypsinized cells and that the fluorescence obtained was not as sharply delineated as with the reference strain AD/169.

FIGURE 4: CMV viruria and 95% confidence limits in different age groups.


No of urines tested
% Positive
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
135
135
135
140
0-6 m
6-12m
1-4 y
5-9 y
age group

All wild CMV isolates were tested by Dr. D. Kennedy, LCDC, Health and Welfare Ottawa, by restriction endonuclease techniques. All isolates produced DNA restriction fragment patterns similar to prototype strain AD 169 when the nucleic acid was digested with the enzyme Eco RI. The pattern was markedly different to that seen when HSV and VZV was digested with the same enzyme.

FIGURE 5: Detection of nuclear fluorescence with strain AD/169 in human fibroblasts (Electronucleonics slides) with CMV monoclone (Biotech) directed against the early nuclear protein, at a dilution of 1:10. Antimouse conjugate (from Microbiological Research Corp.) with 0.005% Evans' Blue diluted 1:10 (Magnification X 438).

COLOURED PICTURES
Images en couleur

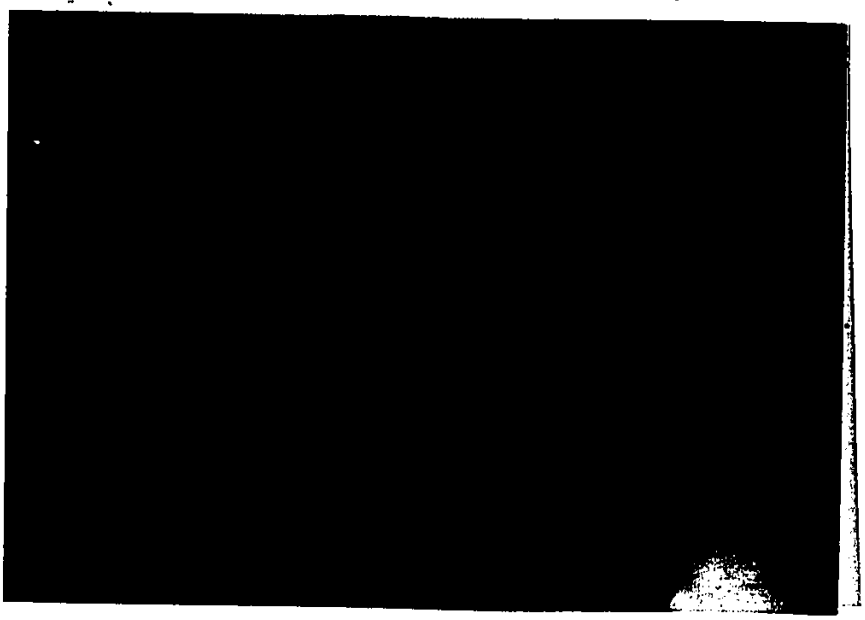


FIGURE 6: Detection of nuclear and cytoplasmic fluorescence with strain AD/169 in human fibroblasts (Electro-Nucleonic slides) with CMV monoclone (Biotech.) directed against the late nuclear protein, at a dilution of 1:10. Same conjugate and magnification as in Fig. 5.

FIGURE 7: Uninfected, trypsinized human fibroblastic cells (MRC5) tested with the early nuclear protein CMV monoclonal antibody. Same conditions and magnification as in Fig. 5.

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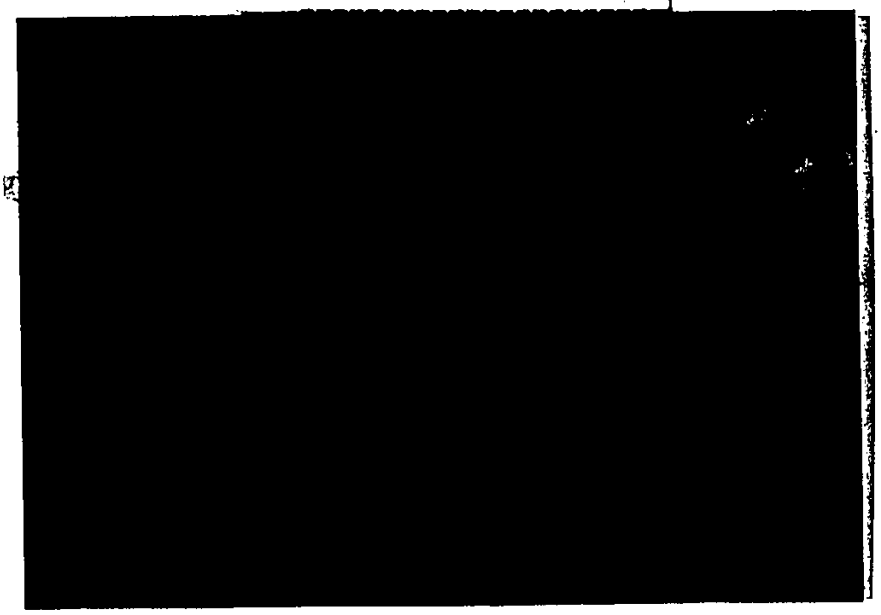

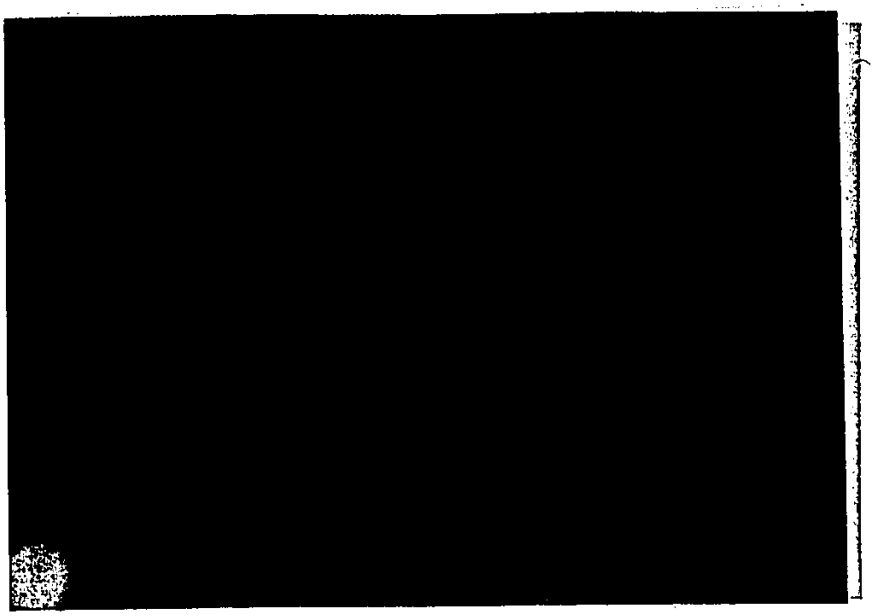

FIGURE 8: HSV1 infected human fibroblasts (Electro-Nucleonic slide) tested with the early nuclear protein CMV monoclonal antibody. Same conditions and magnification as in Fig. 5.

FIGURE 9: HSV2 infected, trypsinized human fibroblastic cells (MRC5) tested with the early nuclear protein CMV monoclonal antibody. Same conditions and magnification as in Fig. 5.

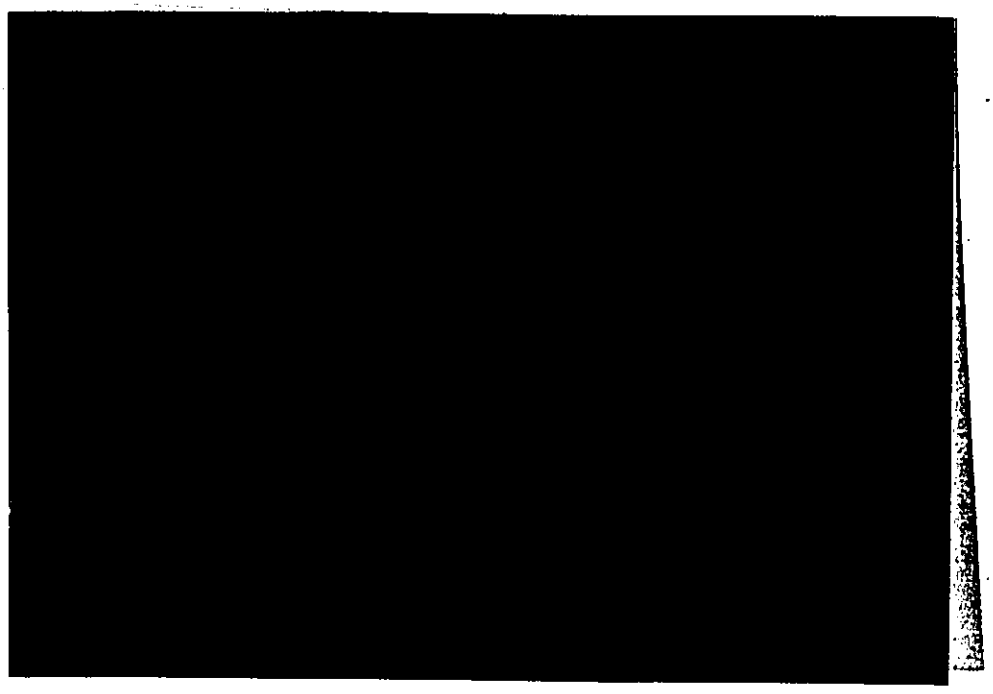

FIGURE 10: EB infected (VCA positive control) lymphoblastoid cells (Electro-Nucleonics Inc.) tested with the early nuclear protein CMV monoclonal antibody. Same conditions and magnification as in Fig. 5.

FIGURE 11: EB infected (VCA positive control) lymphoblastoid cells (Electro-Nucleonics Inc.) tested with the late nuclear protein CMV monoclonal antibody. Same conditions and magnification as in Fig. 5.

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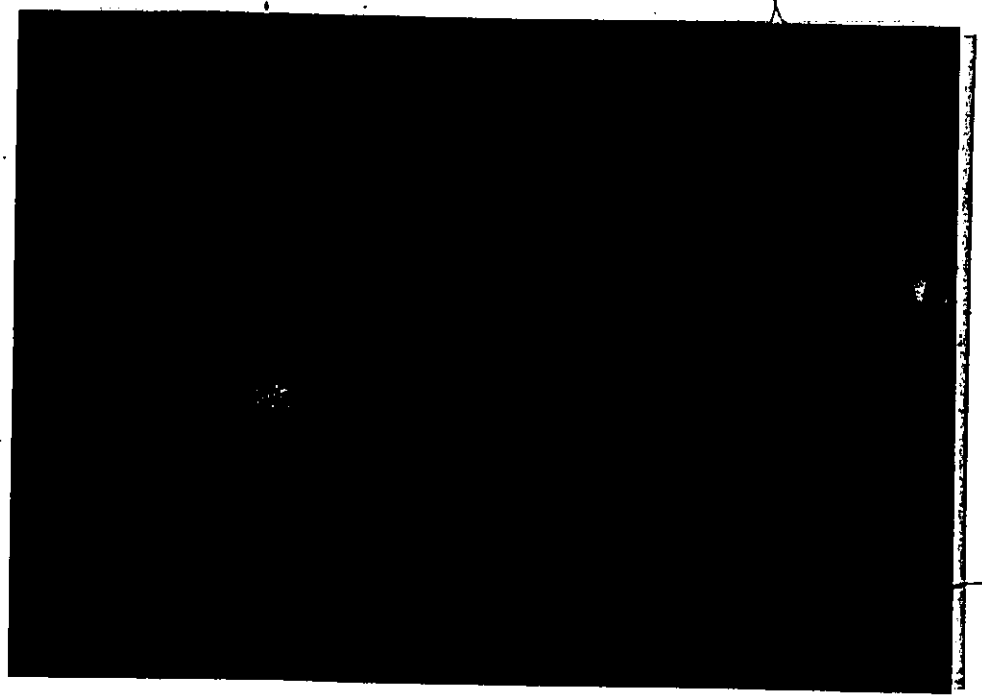
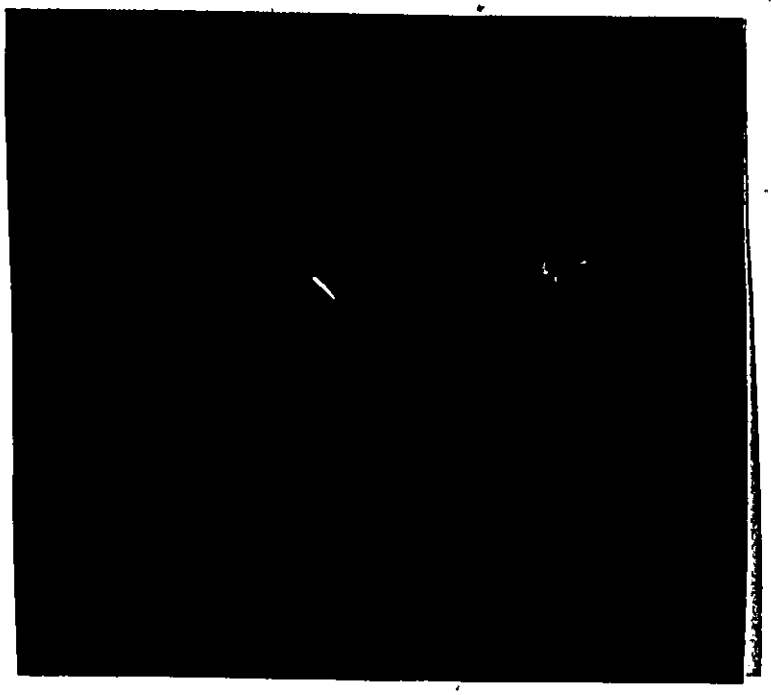


FIGURE 12: EB infected (EBNA positive control) lymphoblastoid cells (Electro-Nucleonics Inc.) tested with the early nuclear protein CMV monoclonal antibody. Same conditions as in Fig. 5.

FIGURE 13: CMV isolate A20, passage 6, tested with late CMV monoclone. Same conjugate and magnification as in Fig. 5.



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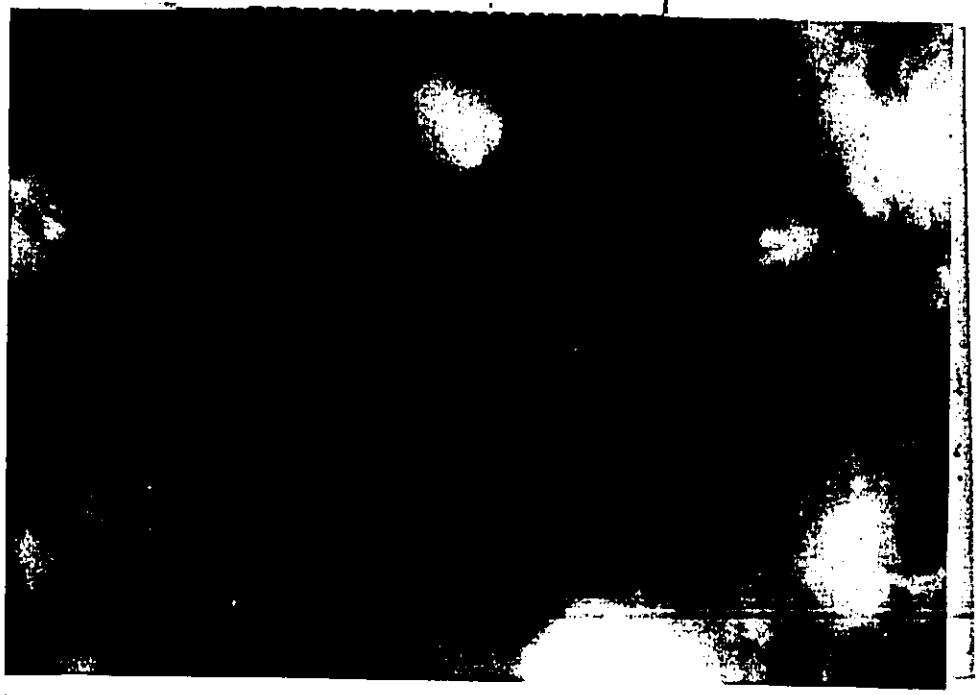
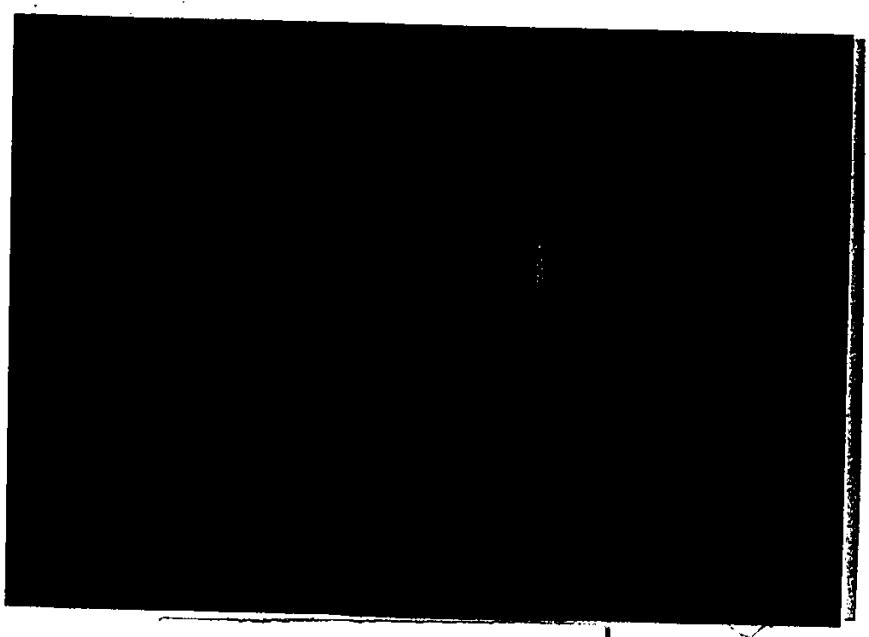


FIGURE 14: CMV isolate B7, passage 5, tested with the late CMV monoclone. Same conjugate and magnification as in Fig. 5.

FIGURE 15: CMV isolate C10 passage 10, tested with the late CMV monoclone. Same conjugate and magnification as in Fig. 5.



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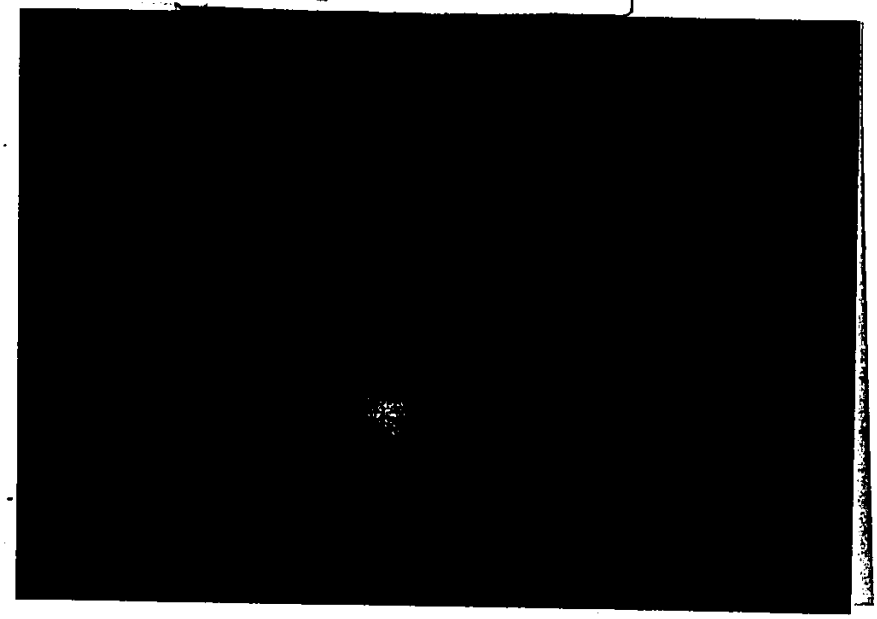

FIGURE 16: CMV isolate C10, passage 7, tested with the early nuclear protein monoclone. Same conjugate and magnification as in Fig. 5.

FIGURE 17: CMV isolate C10, passage 7, tested with the late nuclear protein monoclone. Same conjugate and magnification as in Fig. 5.

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4.2. Serological findings

In order to corroborate our isolation data, a retrospective serological study was undertaken.

Sera received at the Regional Virus Laboratory for diagnostic CMV tests over the period of 6 months from January to June 1982, were analyzed, according to the same age-groups as previously defined for the isolation study. Since these sera had been sent for diagnostic purposes, they were tested at a dilution of 1:20 and above. That means that the seropositivity in each group represents a minimal estimate since antibody at a titer $< 1:20$ would be undetected.

But it should also be pointed out that the population samples used in our serological study were from a patient's population. They don't represent a normal, healthy population. So it may be that if this study had been carried out in healthy people, the complement-fixation antibody prevalence may have been less.

Figure 18 shows the the number of sera tested for CMV antibody, the percentage of positive at a dilution of 1:20 by CFT and the 95% confidence limits.

Figure 18 shows passively transferred antibodies from the mother to the baby in the 0-6 month age-group, then a drop in the 6-12 month age-group. There is no significant difference in the seropositivity rate between the latter age-group and the 1-4 yrs, 5-9 yrs, 10-14 yrs and 15-24 yrs age-groups. A significant rise in the seropositivity rate occurs in the 25-34 yrs age-groups and beyond, indicating that CMV ab acquisition is increasing with age.

In Table XI we show the annual acquisition rate of CMV antibodies (CF 1:20) by age period to see how fast or slow the ab is acquired on an annual basis. The annual acquisition rate has been calculated according to the following formula:

$$\frac{\text{Difference in the percentage of the two groups}}{\text{Time in years between the mid-point ages of the groups}}$$

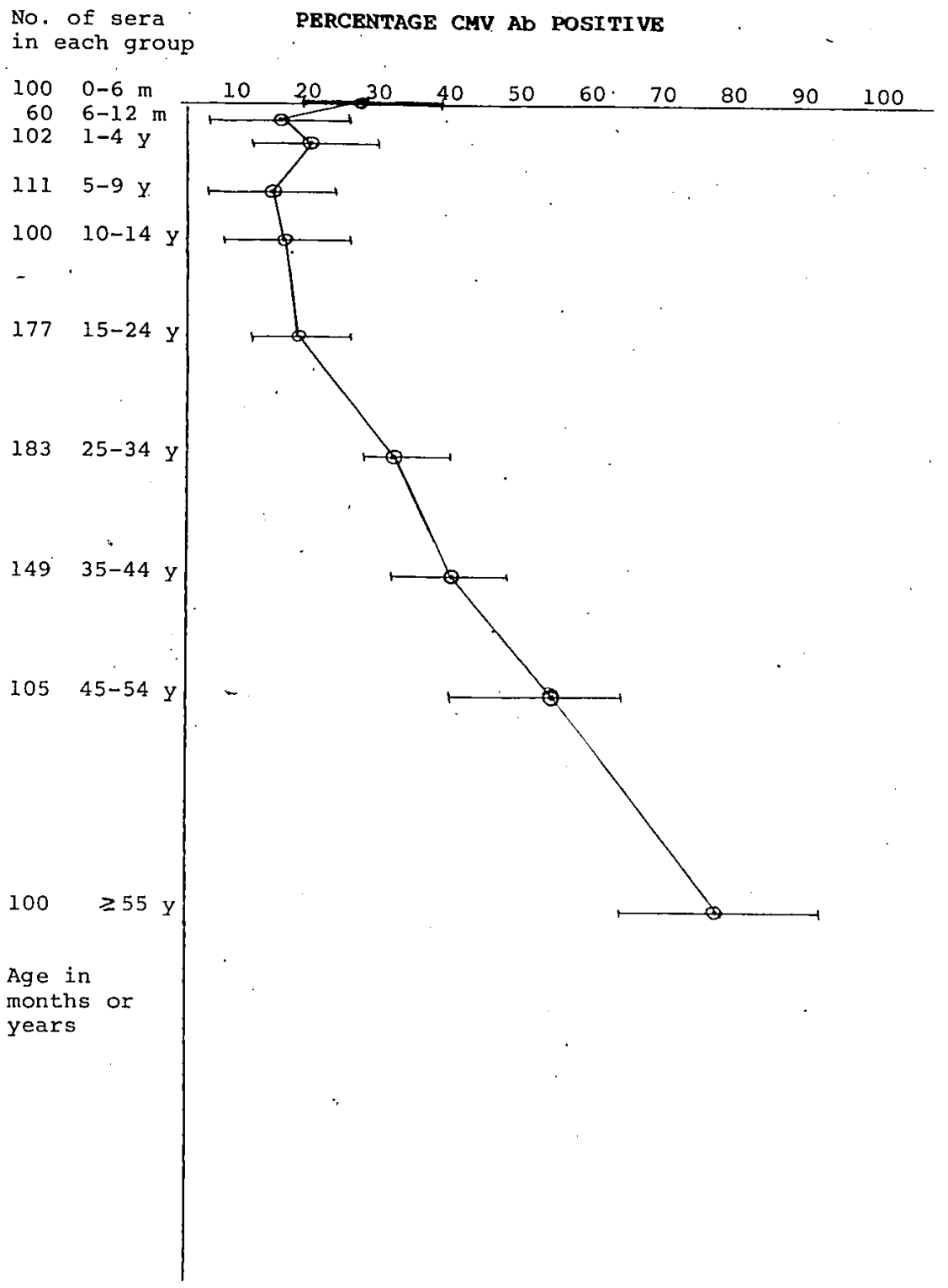
For example, in Figure 18, by taking the percentage of CMV antibody positive in group 0-6 months (which is 27) and the next group 6-12 months (which is 13) and by applying the formula, the annual acquisition rate will be - 28 (mid-point of the first group is 3 months and the second group is 9 months.

$$\frac{13 - 27}{6/12} = \frac{-14}{1/2} = -28$$

Figure 19 is the graphic representation of Table XI and is showing the annual loss or acquisition of CMV antibody in each age group by using the mid point as the point of reference in each age group and graphed to scale on the abcissa.

Figure 19 and Table XI show a very slow annual acquisition of antibody which explains the low isolation findings in Fig. 4. Figure 19 indicates a low of CMV antibodies at the age 3-9 months which corresponds with the low isolation findings in Figure 4 at this particular age.

FIGURE 18: Percentage of CMV antibody positive sera, according to age, by CF 1:20, in sera received at the Regional Virus Laboratory. The vertical bars indicate the 95% confidence limits.

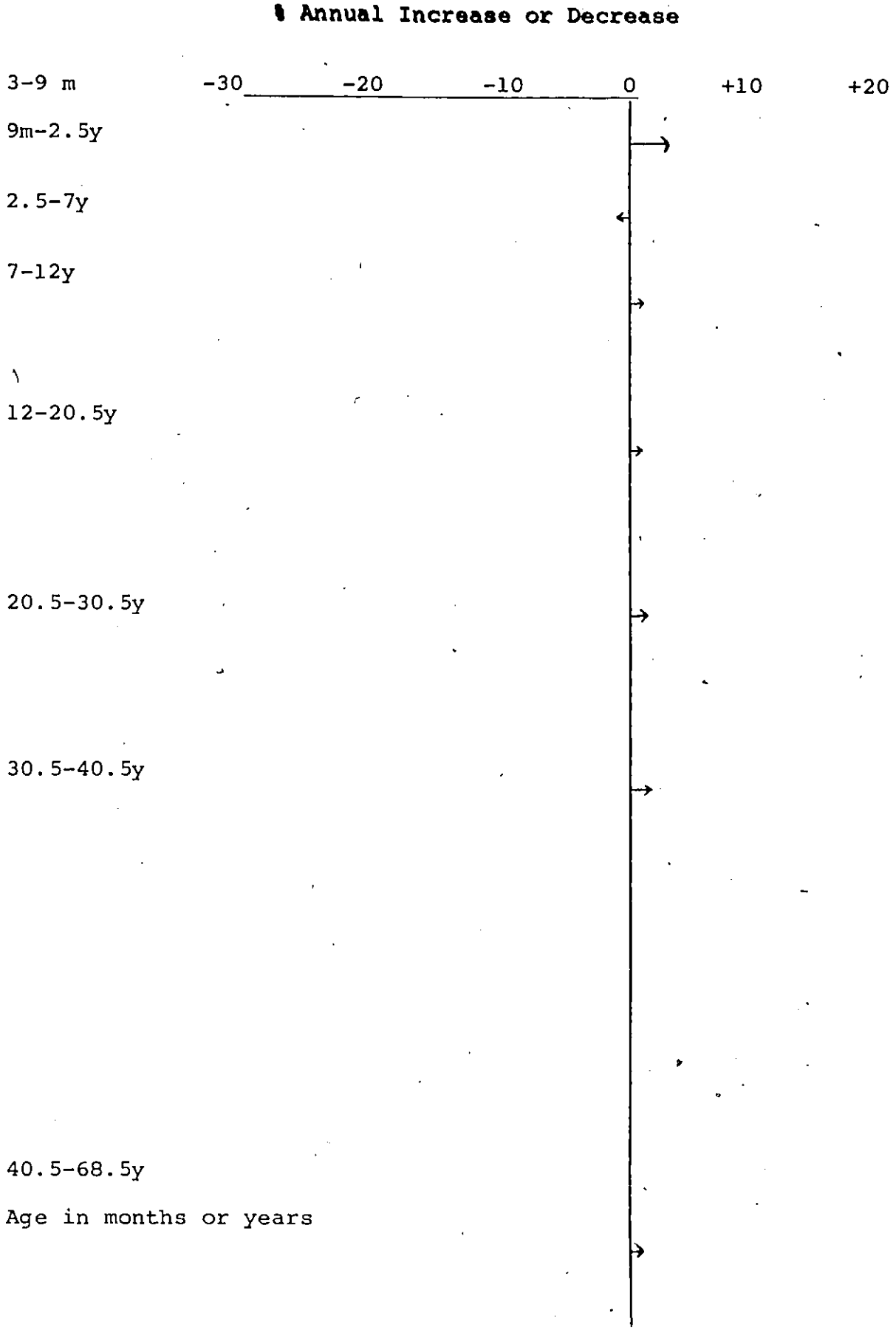

No. of sera in each group
PERCENTAGE CMV Ab POSITIVE
10
20
30
40
50
60
70
80
90
100
100 0-6 m
60 6-12 m
102 1-4 y
111 5-9 y
100 10-14 y
177 15-24 y
183 25-34 y
149 35-44 y
105 45-54 y
100 ≥55 y
Age in months or years

Age Period	*Annual Acquisition Rate (Percent)
3 - 9 mos	-28
9 mos - 2.5 yr	+2.9
2.5 - 7 yr	-1.1
7 - 12 yr	+0.4
12 - 20.5 yr	+0.2
20.5 - 30.5 yr	+0.9
30.5 - 40.5 yr	+1.4
40.5 - 68.5 yr	+1.4

TABLE XI: Percent Annual Acquisition Rate of CMV Antibody (CF 1:20) By Age Period*

* = $\frac{\text{Difference in the precentage of the two groups}}{\text{Time in years between the mid-point ages of the groups}}$

FIGURE 19: Percent Annual Acquisition Rate of CMV Antibody


% Annual Increase or Decrease
3-9 m
-30
-20
-10
0
+10
+20
9m-2.5y
2.5-7y
7-12y
12-20.5y
20.5-30.5y
30.5-40.5y
40.5-68.5y
Age in months or years

5. DISCUSSION

The general objective of our research was to study the prevalence of HCMV in our region. The results of the isolation study show no CMV isolate above the age of 9 years and show a high peak at the age of 1-4 yrs (Table IX and Figure 4). The incidence of CMV excretion appears to be highest in small children, and then the cytomegaloviruria decreases with age. The rare findings of these viruses in the urines of older children and in adults may be explained by immunity developing with age or may be related to undetermined systemic or local resistance factors.

In planning this investigation, it was expected that the proportion of CMV excreters in any age-group would be small. It was argued therefore that as long as the pools of urines were kept small, it could be expected with reasonable certainty that any positive pool would contain no more than one excreter. This assumption is confirmed being correct on the basis of the following data and arguments, which treat the groups in order of proportions of positives. The full argument as generated by Mr. P.H. Phipps, Laboratory Scientist at the Regional Virus Laboratory at the CHEO, is given for group C and conclusions by the same argument given for groups B, A and D.

Argument for 1 excreter per pool

Group C:	No. of pools tested	31
	No. of pools positive	10
	Proportion positive	.32
	Total No. of subjects	155

Assume that the CMV excreters were encountered randomly

For u = .3, the Poissonian distribution predicts that:

74% of the pools will contain 0 excreter

22% " " " 1 "

3.3% " " " 2 " 's

Furthermore, the probability that one pool contained more than one excreter is .03, and the probability that two or more pools contained more than one excreter is $\leq 0.0372 = \leq 0.0014$.

- By similar argument, the probability that there were 10 excreters among the 155 subjects is .963; the probability that there were 155 excreters rather than 10 is .033, and the probability that there were more than 11 rather than 10 or 11 is .004.

By similar argument, it can be shown that: In group B where 5 out of 25 pools, each of 5 individuals, were positive; the probability that there were five excreters among the 125 subjects is .983; the probability that there were 6 rather than 5 is .016 and the probability that there were 7 or more rather than 5 or 6 is .001.

And: In group A where 2 out of 25 pools, each of 5 individuals, were positive; the probability that there were more than 2 excreters among the 125 individuals is $< .005$.

Also: In group D: Where 2 out of 32 pools, each of 5 individuals, were positive; the probability that there were more than 2 excreters among 160 individuals is $< .002$.

The hypothesis that there were more than one excreter in any of the pools tested can, therefore, be rejected ($p < 0.04$).

We are therefore justified in assuming that any positive pool represents one single excreter out of the 5 constituting individuals and Table IX a shows in the last column the percentage of positive individual urines in each age-group.

The results of several studies concerning CMV excretion (20, 122) (described in 2.2.2.) indicate that the primary infections almost invariably occur in small children. Among normal adults viral excretion in urine was only observed in pregnant women (40).

Figure 18 illustrating the findings of the retrospective serological study shows a peak of antibody at the age of six months which could be explained by the acquisition of maternal antibodies through placenta or breast milk. This last hypothesis is corroborated by the data obtained by Phipps and Rossier (125), which showed a CF seropositivity rate of 27.5% in pregnant women sera in the first trimester of pregnancy. In the 0-6 month age-group in our study, it was 27%. The serum antibodies decrease by 12 months of age and another peak at age of 1-4 year appears, probably demonstrating CMV activity during early childhood. There is an apparent drop in the percentage of positive subjects after age 4 which is most probably the result of sampling error.

CMV excretion in urine shows a peak in the 1-4 year age-group (Figure 4) which parallels the prevalence of CMV antibodies in this particular age-group (Figure 18).

The complement fixation test which has a high specificity (99%) and a high false negative rate (25%) may have given underestimated positive serological results due to its low sensitivity.

Table XI and Figure 19 where the mid point is the point of reference, show an antibody loss in the 3-9 month age-group which corroborates Phipps and Rossier data and is due to the physiological loss of maternal antibodies in this age-group (95).

Our serological findings show that primary infections may also occur in older children and in adults who possibly excrete CMV in amounts which are not detectable in cell cultures. The spread of CMV is also significantly influenced by socioeconomic factors; this was confirmed in many studies (69, 85, 88, 33, 1, 74, 122) (described in 2.2.3.).

TABLE XII: Cytomegaloviruria in unselected pregnant women in different areas in the United States.

TABLE XIII: Incidence of cervical cytomegalovirus infection during pregnancy in different areas in the United States.

TABLE XIV: Cytomegaloviruria in unselected hospital admissions.

Location	Year	Number Tested	Number Positive	Percent Positive
Pittsburg, Pa (84)	1972			
Negro		76	2	2.6
Caucasian		28	1	3.6
Rochester, N.Y. (39)	1973	209	9	4.3
Birmingham, Ala (99)	1973	463	29	6.3

Location	Year	Number Tested	Number Positive	Percent Positive
Birmingham, Ala (99)	1973	404	48	11.9
Pittsburg, Pa (84)				
third trimester	1972	23	2	8.7
Navajo reservation, Ariz (84)	1972	26	4	15.4
third trimester				

Location	Age Range (years)	Number Tested	Number Positive	Percent Positive
Rochester, N.Y. (37)	birth-14	100	1	1
Houston, Texas (6)	2-10	30	6	20.0

Age of subject	No. positive/No. tested	Percent positive
Cord blood	12/17	71
6 weeks	5/17	29
6-23 months	3/21	14
2-4 years	11/36	31
5-9 years	11/33	33
10-15 years	22/49	45
18-25 years	52/98	53
> 35 years	42/52	81

TABLE XV: Prevalence of complement-fixing antibodies in Washington, D.C. (Data from Rowe et al (1956) (104).

Numerous studies of CMV epidemiology have been carried in the United States and are summarized in Tables XII to XV.

The above United States data when compared with the Canadian data in Hamilton, Montreal and Ottawa show that the rate of excretion of CMV in neonates, in children, in young adults and in pregnant women is much less in Canada. Noteworthy, the prevalence of complement fixing antibodies in Albany and Houston are 45% and 79% respectively as shown in Table III (69).

These data explain why the rate of CMV infections in renal and bone marrow transplant patients is less in Canada than in the United States (17, 47, 11, 82). The reasons for this difference must be found probably in the socio-economical factors which prevail in Canada. Such factors have been shown to be important in the dissemination of CMV infection.

Tables XVI, XVIII, XIX show unpublished data kindly provided by Dr. Ranko, Institut Armand Frappier, Montreal, who carried out studies similar to ours. The percentage of cytomegalovirus seropositive pregnant women during the first trimester of pregnancy in Montreal Hospitals is 30.5 (Table XVI and Table XVIII) and at the Ottawa General Hospital (95) is 34.9 (Table XVII). These results are almost identical, considering that the CF were performed in Montreal at 1/8 and at Ottawa at 1/5.

By comparing Ottawa serological data (Table XVII) with that of Montreal (Table XVI), we conclude that the prevalence of CMV infections in both cities is low among the pregnant women in the first trimester of pregnancy.

In the Montreal region, the isolation rate of CMV from the cervix was 3.5% and 1.7% in urines of pregnant women in the last trimester of pregnancy (Table XIX). In our study, the isolation rate of CMV was nil in both urines and cervical secretions of pregnant women (Table IX a, IX c), but that difference may not be statistically significant.

A study in Hamilton, Ontario (Department of Paediatrics, Division of Neonatology (9) showed that out of 13,183 newborns, only 58 excreted (0.44%) CMV. At this age in our study (Table IX a), 1.6% of the urines were positive, but our age-group was broader, extending from 0-6 month while in Hamilton, only newborns were studied.

TABLE XVI: Percentage of cytomegalovirus seropositive pregnant women during the first trimester of pregnancy in Montreal hospitals.

TABLE XVII: Percentage of cytomegalovirus seropositive pregnant women during the first trimester of pregnancy at the Ottawa General Hospital (95).

Hospital	Number of pregnant women	Seropositives* Number	%
Hôpital Général Fleury	2 023	607	30,0
Hôpital Ste-Justine	887	316	35,6
Centre Hospitalier Maisonneuve-Rosemont	155	53	34,1
Hôpital Charles Le Moyne	1 504	420	27,9
TOTAL	4 569	1 396	30,5

* by complement fixation 1/8 is considered positive

CF titer	Negative	Positive 1:5 - 1:40	1 1:80
NO	468	197	52
%	65.3	27.5	7.2
NO fractionated			52
NO IgM positive			1

TABLE XVIII: Percentage of seropositive pregnant women and geometric mean titers of complement fixing antibodies to cytomegalovirus by age, in Montreal hospital.

TABLE XIX: Isolation of cytomegalovirus from the cervix and urine samples in pregnant women from the region of Montreal.

Age group (yrs)	Number of sera	Seropositives		Geometric mean of CF titers	95% confidence limits
		Number	%		
15-20	470	118	25,1	21.46	19.40-23.74
21-45	1758	449	25,5	20.17	19.04-21.37
26-30	1652	511	30,9	20.73	19.74-21.77
31-35	575	262	45,7	19.56	18.32-20.89
36 and over	114	56	49,1	17.67	15.37-20.30
TOTAL	4569	1396	30,5	20.26	19.65-20.88

	Number of pregnant women	Trimester 1st (%)	2nd (%)	3rd (%)	Total No. of positive cases	%
Cervix	1427	9/1321*(0.7)	2/212(1)	5/144(3.5)	16	1.1%
Urine	966	0/948(0)	0/164(0)	2/116(1.7)	2	0.2%

* Number positive/number tested

6. CONCLUSIONS

Objectives 1 and 3 of our study were fulfilled by determining the frequency of CMV urinary excretors in age-groups from zero to eighty years, and by studying the rate of cervical CMV excretion in healthy pregnant women during the third trimester of pregnancy. We show that the incidence of CMV infections in the Ottawa-Carleton region is low. This may be due to the high socioeconomic status of the region.

In this Canadian community, cytomegalovirus excretion is rare and appears to be confined to young children.

The extremely low incidence of viral excretion in the adults is corroborated by the results of cervical swabs which have been tested. It explains the fact that in ten years at the Children's Hospital of Eastern Ontario, only four cases of congenital cytomegalovirus infection have been diagnosed, although not confirmed serologically or by isolation of CMV at the time of birth.

Objective 2 was fulfilled by studying the acquisition of complement-fixing antibodies. It clearly indicates that primary infections may also occur in older age-groups. Such individuals possibly excrete CMV in amounts which are not detectable in cell cultures.

Our study confirms that the incidence of CMV infections in Ottawa-Carleton is very low and different from other regions of North America.

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