Cytomegalovirus Infections with Reference to Isolations from Lymph Nodes and Blood.* (31651)

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Since the first isolations of human cytomegalovirus (CMV)(1-3), this agent has been shown not only to be the cause of generalized cytomegalic inclusion disease(4,5), but also has been found in normal individuals(2,6,7), as well as in children with neoplastic and other diseases(7-9). The present investigation was prompted by our observation of recurrent cytomegalic inclusions in a patient with autoimmune acquired hemolytic anemia (AHA)(10). Repeated isolations of CMV from biopsied lymph nodes indicated it to be involved in a generalized lymphadenopathy which paralleled hemolytic episodes. This report is concerned with studies of similar and related cases which resulted in the isolation of CMV from peripheral lymph nodes and blood as well as from other sources.

Materials and methods. Cell cultures. A strain of human fibroblast-like cells, Det.-532 (11), was employed. A mycoplasma-free seed stock of this strain in its ninth culture passage was preserved at —190°C(11) and these cells (or their progeny within a few passages) were used throughout this study. Monolayers were prepared in tubes, and in glass or plastic flasks, by seeding with 10^5 or 10^6 cells/ml in minimal essential medium (MEM)(12) 90%, fetal bovine serum 10%, 2 g/l $NaHCO_3$, and with antibiotics (penicillin, 100 U/ml; streptomycin, 100 μg/ml). These were incubated in a 5% CO_2-95% air atmosphere at 36-37°C, for 2 days, and then tubes and flasks were stoppered. Monolayers were formed in 2-4 days depending on the cell density of the inoculum. MEM with 2% fetal bovine serum was used for maintenance of cultures over prolonged periods (usually about 1 month). Experience with a known cytomegalovirus (C87 strain provided by Dr. J. L. Melnick, Baylor University) indicated that CMV multiplied in Det.-532 cells

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TABLE I. Disease Categories of Patients Studied for Cytomegalovirus Infections.

Diagnosis	No. of patients	Cytomegalic inclusions	Virus isolated
Hemolytic anemia with lymphadenopathy	8	4	2 CMV
Hemolytic anemia	2		
Lymphadenopathy-lymphadonitis	7		
Hepatitis	7	2*	2 CMV*
Post rubella syndrome	5		4 rubella†
Leukemia	2		
Carcinoma	1		
Lymphoblastoma	1	1	
Hodgkin's disease	1		1 adeno-like
Rabdosarcoma	1		
Mast cell disease	1		
Thrombocytopenic purpura	1		
Congenital CID	1	1	1 CMV
Unknown	4		
Totals	42	8	10

* Virus and inclusions in 1 patient.
† Isolated by Dr. R. Brackett, Research Laboratories, Parke, Davis and Co.

in a manner similar to that described in the literature (13-15).

Specimens studied. Over a 1-year period 42 infants and children ranging in age from newborns to 15 years with the clinical diagnoses listed in Table I were studied. Portions of biopsies obtained for histopathologic study were examined for evidence of CMV. Biopsies of lymph nodes and liver as well as heparinized bone marrow and blood, and urine were obtained. Autopsy specimens consisted of lymph nodes, liver, lung, kidney, spleen, and salivary gland. Specimens were processed for virus isolation immediately after collection unless otherwise indicated. In a few instances, specimens were stored frozen (—20°C or —190°C).

Virus isolation procedures. All solid tissues were minced to yield particles approximately 1-2 mm^3 in size. The constituent cells of the

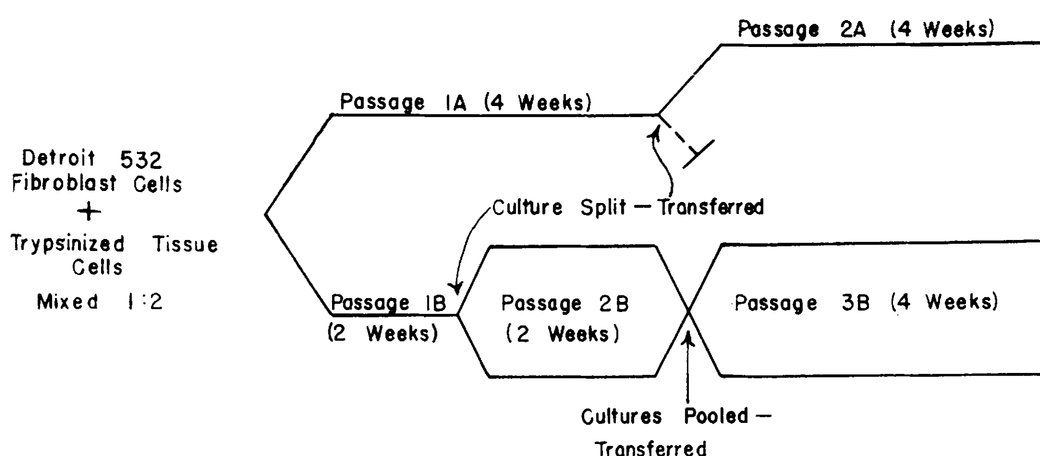

FIG. 1. Diagrammatic scheme of inoculations, cell cultures, transfers, and passages used for isolation of cytomegalovirus from specimens.

tissue fragments were then dispersed by trypsinization according to procedures described by Wallis *et al*(16) for preparation of kidney cell cultures, and the resultant cells suspended in MEM containing 10% fetal bovine serum and antibiotics. These cells were mixed with trypsinized cultured fibroblasts in proportions of 1:2 in a manner similar to that employed by Benyesh-Melnick *et al*(7) for continuous passage of CMV isolates. The cell mixtures were inoculated into tubes, small plastic flasks, and petri dishes containing coverslips, and incubated in a 5% CO_2-95% air atmosphere until mixed monolayers had formed. The tubes and flasks were then stoppered and incubated with changes of medium (weekly) consistent with maintaining the integrity of the monolayers.

All flasks were observed daily for cytopathic changes (CPE) indicative of viral infection. These cultures, with or without CPE, were subcultured by trypsinizing cell monolayers and cells were resuspended in the previously removed culture fluids. The suspensions were mixed with fresh tissue culture cells and again grown and observed as described. In instances where no viral effects were observed in the original cultures, 3 blind passages were carried out using 2 culture flasks per passage. This isolation procedure is outlined in Fig. 1.

While the buffy coat layer was handled in the manner described above, plasma, serum, and urine were adsorbed directly onto the Det.-532 monolayers (0.25 ml per monolayer culture) and incubated for one hour after which media was added. Subsequently, they were observed and handled identically to the "mixed-cell" cultures.

CMV isolation. Recognition of initial and advanced cytopathic changes characteristic of CMV was made by low power observation of monolayers in flasks, or by observation of Bouin-fixed and hematoxylin-eosin stained monolayers from coverslip cultures prepared with each passage series. Focal areas of greatly enlarged and rounded cells among the fibroblastic networks were typical of CMV-infected cultures(16). Upon staining, these cells always showed large, irregular intranuclear inclusions with margination of the chromatin, and cytoplasmic inclusions less regularly. Cultures exhibiting such cytopathic changes were trypsinized and mixed with suspensions of fresh or thawed Det.-532 cells and the mixture seeded in flasks. In this manner virus isolates were transferred one or more times until the focal changes appeared earlier and became more generalized. These could then be preserved by freezing trypsinized infected cells using the same procedure as that employed for uninfected cultured cells(11). When needed for virus seed, the infected cells were thawed rapidly and virus was propagated by mixing with fresh or thawed suspensions of Det.-532 cells.

The virus isolates were characterized as CMV mainly by the production of typical focal necrosis and intranuclear and cyto-

TABLE II. Specimens Examined for CMV or Cytomegalic Inclusions.

		No. of specimens		
Specimen	No. of patients‖	Tested	Contaminated	Yielding CMV / Exhibiting inclusions
Lymph node (B)*	27	35	3	3/7
" " (A)†	1	1		
Blood‡	24	30		
" (BCL only)§	7	7	1	1/—
Urine	27	63	24	1/—
Marrow	16	16	1	
Liver (B)	7	7	1	1/0
" (A)	2	2		1/1
Lung (A)	2	3		1/2
Kidney (A)	2	2		
Spleen (A)	4	4		1/1
Salivary gland (A)	1	1		
Placenta	2	2		
Totals	—‖	173	30	9/11

* (B) = biopsy specimen.
† (A) = autopsy specimen.
‡ Plasma, red cells, and buffy coat layer were tested separately.
§ Only buffy coat layer was tested.
‖ Different or multiple specimens from 42 patients.

plasmic inclusions in cultured human fibroblastic cells. In addition, the isolates failed to multiply in HeLa cells, primary rabbit kidney monolayers, and in CA membranes of chick embryos, in contrast to herpes simplex controls, and were sensitive to chloroform. Three of the isolates were found to be typical of CMV by electron microscopy of thin sections of infected Det.-532 cells by A. J. Dalton, National Cancer Institute.

Results. From September 1964 to October 1965, a variety of biopsy and autopsy specimens as well as blood and urine was examined for comparative histologic and virologic evidence of cytomegalovirus infections. A total of 173 specimens (Table II) was tested from 42 patients with the clinical-pathological diagnoses listed in Table I. As shown in Table II, 30 of the 173 specimens exhibited fungal contamination in the primary tissue cultures and thus could not be used for virus isolation. Of the 143 remaining specimens, cytomegalic inclusions were observed in 11 obtained from 6 patients; from 5 of these 6 patients, 9 CMV isolates were obtained.

Virologic, histologic, and clinical data on the 5 individuals yielding CMV are given in Table III. The first, *B.W.*, was a 12-year-old Negro male who had had over 35 major hemolytic crises concurrent with generalized lymphadenopathy since the age of 3 and who had been clinically diagnosed as an acquired hemolytic anemia of the autoimmune type. Biopsies of lymph nodes since age 7 had retrospectively shown typical cytomegalic inclusions, and the isolation and identification of CMV from a lymph node obtained in September 1964 led to further virologic studies. Over a period of a year, 4 lymph node biopsies (3 exhibiting typical CMV inclusions in varying numbers), 3 blood specimens, 4 urine specimens, spleen (obtained during splenectomy), bone marrow, and autopsy specimens were cultured from B.W. Table III shows that CMV was isolated from 2 of the lymph node biopsies collected at widely separated intervals, and from a urine specimen. Material from the first lymph node specimen produced a CMV-type CPE as early as 11 days. The second, obtained 8 months later yielded CMV in each of 2 series of cultures in second passage, 28 and 39 days respectively after inoculation of the primary culture. The positive urine specimen yielded CMV in primary culture 32 days after inoculation. The

TABLE III. Clinical, Histopathological, and Virological Observations in Five Patients Yielding Cytomegaloviruses.

Age	Clinical and/or histopathological diagnosis	Specimens: Tissue or fluid	Date tested	Virologic findings: Cyto-megalic inclusions	CMV isolation	CPE in cu Passage	Tc
12 yr	Acquired hemolytic anemia, autoimmune	Liver	1959, 60	0	ND		
		Lymph node	1961	+	ND		
		" "	1964 (6/15)*	+	0		
		" "	" (9/22)	+	+	1 a§	
		" "	1965 (3/25)*	ND	0		
		" "	" (5/24)	+	+	2 a,b	
		Spleen	" (3/25)	0	0		
		Blood	" (1/25, 3/19, 5/20)	ND	0		
		Bone marrow	" (3/25)*	ND	0		
		Urine	1964 (10/22)†	0	+	1 a	
		"	1965 (3/22, 5/3, 5/28)	0	0 or contam.		
		Autopsy tissues	1965 (10/8)	0	0		
1 mo	Acquired hemolytic anemia	Lymph node	1964	?	+	1 a,b	
4 mo	Hepatitis (giant cell type)	Blood (BCL)	1965 (1/15)	ND	+	2 a 3 b,c	
		Urine	" "	0	contam.	1 a	
		Lymph node	" (1/20)	0	0		
		Liver	" (1/27)	0	0		
		Bone marrow	" (1/19)	0	0		
3 mo	*Idem*	Liver	" (6/17)	0	+	1 a 2 b	
		Lymph node	" "	0	0		
		Urine	" (6/14)	0	contam.		
		Blood	" (6/9)	ND	0		
		Bone marrow	" (6/9)	0	0		
1 day	Congenital cytomegalic inclusion disease	Liver	" (7/6)‡	+	+	1 a,b,c	
		Lung	" (7/6)	+	+	1 a,b	
		Spleen	" "	+	+	3 b	
		Lymph node	" "	0	0		

ed by course of predisone treatment.
ed by course of nitrogen mustard treatment.
at —20°C; cultured 7/20.
meral refers to the number of passages. The letters a, b or c refer to the passage series.

other specimens yielded no viruses.

Patient *Par.* was a premature infant born with a maculopapular rash, normoblastemia, generalized lymphadenopathy, and evidence of hemolysis by a drop in hemoglobin of 20.0 to 5.5 g% in 11 days. A lymph node biopsy obtained at 20 days of age revealed one possible cytomegalic inclusion after a long search. Two culture flasks, inoculated with cells from this lymph node together with Det.-532 cells as described, each exhibited an initial cytomegalic CPE at 23 and 35 days, respectively.

Patient *Hal.* was a 4-month-old infant with hepatitis, first noted at 2 weeks of age which increased in intensity. Hemolytic anemia was also evident. A liver biopsy showed a few typical cytomegalic inclusions. Although liver, lymph node, bone marrow, and buffy coat layer of blood were cultured for CMV as described, only the buffy coat layer and urine yielded evidence of virus. In the primary passage inoculated with buffy coat layer no evidence of infection was observed. However, cytomegalic lesions were first noted in the second and third blind passages after 45 and 26 days, respectively (each was 66 days after the start of the initial culture), and in the third blind passage after 26 days (77 days after the initial seeding) in a second culture series from the same specimen. The possibility that CMV was also present in a urine specimen obtained at the same time as the blood was indicated by characteristic CPE on the thirteenth day of primary culture, but fungus contamination prevented an isolation.

Patient *Ty.* was a 3-month-old infant with neonatal hepatitis and lymphoid hyperplasia. Although biopsies did not reveal classical cytomegalic inclusions, CMV was isolated from each of 2 series of cultures from the liver specimen. One exhibited typical focal CPE on the 26th day of the primary culture, while the other revealed CPE in second passage in each of 2 cultures following an initial blind passage. Cultures from lymph node, bone marrow, urine, and blood were negative.

Patient *Osb.* was an infant born with the characteristic symptomatology of generalized cytomegalic disease, *i.e.*, scattered petechiae, thrombocytopenia, and hepatosplenomegaly. This infant expired within 24 hours of life and specimens of liver, spleen, lung, and lymph nodes were cultured as described. As shown in Table III, lung and liver which had numerous cytomegalic inclusions produced typical CPE on 7 and 13 days, respectively, in the primary mixed cultures. Spleen, which had fewer inclusions, produced CPE on the 5th day of culture in the third passage, or 34 days after the initial seeding.

In 2 additional cases, CMV-type inclusions were observed in the lungs of a 10-year-old boy who expired with autoimmune acquired hemolytic anemia, and in a lymph node biopsy of a 1-month-old infant with hepatitis. In neither case could CMV be isolated from these, from other tissues, or from urine.

Discussion. In the course of these studies, CMV was isolated from enlarged lymph nodes in 3 instances and from blood in one. In 2 lymph nodes from *B.W.*, characteristic inclusions were abundantly present. In addition, numerous smaller cells with Feulgen positive cellular lesions were observed which were analogous to the developmental stages of inclusion bodies described in infected tissue culture cells(13). Analysis of the latter by fluorescent antibody techniques and electron microscopy is in progress. However, wherever comparable observations could be made, virus isolations paralleled the presence of classical inclusions with the exception of two instances.

It is of interest that the lymph nodes of each of the first 2 cases in Table III *(B.W., Par.)* exhibited lesions and virus indicating R.E. system infections in parallel with some form of hemolytic anemia. CMV could be a primary cause of disease in *B.W.* who had autoimmune (intermittently Coombs positive) acquired hemolytic anemia. *Par.* had lymphadenopathy and acquired hemolytic anemia of the non-autoimmune type, but familiar clinical manifestations of CID of the newborn such as encephalitis or hepatitis were lacking. In the 2 cases of neonatal giant cell hepatitis *(Hal., Ty.)*, CMV inclusions were not observed in lymph nodes or liver biopsies. However, the liver biopsy of one *(Ty.)* did yield virus and evidence of CMV was found in the urine and isolated from blood of the other *(Hal.)*. These findings further support the role of this agent as a cause of neonatal hepa-

titis(18). The classical picture of congenital generalized CID of the newborn was apparent in only one case *(Osb.)*. The tissues examined from this patient, with the exception of lymph nodes, contained easily recognizable cytomegalic cells. The liver contained sufficient virus to be isolated within a short period even after storage at —20°C.

While viremia has not previously been reported in human CMV disease, it has been thought that viremia must account for CMV in multiple organs and presence of inclusion-bearing cells or virus in urine(19,29). Thus the isolation from blood is noteworthy, although it is unfortunate that only the buffy coat layer was tested in this instance and it is not known whether free virus was associated with other blood components. However, because this was the first known isolation of CMV from blood, it is important to note that laboratory contamination was precluded by the fact that other than these cultures, no CMV was being cultured or isolated in the laboratory during that particular period.

The isolation procedures were modified from the technique described by Rapp *et al* (14) for the routine maintenance of CMV. By mixing trypsinized tissue cells with suspensions of cultured fibroblasts, homogenization of the tissues was avoided, thus minimizing the possible loss of CMV which may be present in small concentrations and which is known to be relatively labile. The handling of tissues as well as the type of cell cultures used were directed toward the isolation of cytomegalovirus. A strain of human fibroblast-like cells was found suitable for the isolation procedures described. It was susceptible to strains of CMV, cultures could be maintained for long periods, and replicate cell populations were available for all of the isolation procedures described. However, while such cell strains are susceptible to a variety of passaged viruses, they may not readily detect naturally occurring viruses when isolation attempts are being made. Thus, viruses more readily isolated in other types of cells might have been missed if present.

Summary. A search for evidence of cytomegalovirus (CMV) infection was made in a group of patients with hemolytic anemia associated with lymphadenopathy as well as several other diseases in which CMV infections often occur. A total of 173 specimens from 42 patients with a variety of diseases yielded 9 CMV isolations from 5 of 6 patients exhibiting CMV inclusions. The 5 patients included 1 with autoimmune hemolytic anemia, 1 with acquired hemolytic anemia of the non-immune type, 2 with neonatal hepatitis and 1 with congenital cytomegalic inclusion disease. CMV was isolated from biopsied lymph nodes of both hemolytic anemia patients and variously from blood, urine, liver, lung, and spleen of the 5 patients depending on the condition, time, or the disease of the patient involved.

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