Cytomegaloviruria in Children with Acute Leukemia and in Other Children.*† (29654)

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Cytomegalovirus can be readily isolated from children with generalized cytomegalic inclusion disease(1,2). Hanshaw and Weller (3) reported on the recovery of cytomegalovirus from urines of children with various malignancies (4 positive of 50 children tested). This study and the reports of others (4,5) on cytomegalo-type inclusions found in autopsy material of leukemic patients led to the assumption that cytomegalovirus had existed in a hidden form during life. Rowe and coworkers(6) followed a closed (nursery) population of children, 8 months to 4 years of age, in which upper respiratory viral infections were continuously circulating. Of the mouth swabs tested from 47 children in this large "family," 13 were positive. Eight of these 13 children were tested for viruria and 7 were positive. No virus was found in mouth swabs of 108 newborn infants and 26 adults chosen at random for testing. Others (7,8) have also reported on failure to isolate cytomegalovirus from infants without cytomegalic inclusion disease.

In our laboratory a search for viruses in the urine was carried out as part of an extensive study of children with acute leukemia (9,10). We did not find significant differences in the incidence of cytomegaloviruria among 42 children with acute leukemia, 30 children with diseases other than leukemia and 29 normal control children studied. The results of cytomegalovirus isolations from the urines of these 3 groups of children as well as from infants with cytomegalic inclusion disease are discussed here.

Materials and methods. Tissue cultures. Local strains of human embryo lung (HEL) fibroblasts, carried in serial passages, were employed throughout the study. Monolayers grown in 16 oz bottles were treated with 0.2% trypsin and the resulting cell suspension was spun at 800 rpm for 10 minutes. Cells were resuspended in Eagle's medium with 20% calf serum and 0.75 g $NaHCO_3$ per liter and were seeded in stationary culture tubes at a concentration of 10^5 viable cells per tube. Monolayers were formed within 24 to 48 hours incubation at 37°C. Eagle's medium with 5% fetal bovine serum and 3 g $NaHCO_3$ per liter was used for cell maintenance. All media contained penicillin (100 U/ml) and streptomycin (100 μg/ml).

HeLa cells were also utilized. They were carried in serial passage as the HEL cells. Tubes were seeded with 50,000 cells per tube and were maintained as the HEL cells but 2% inactivated calf serum was used in the medium instead of 5% fetal serum.

Specimen collection and testing. Urines were collected in sterile glass containers and sent immediately to the laboratory. Most of the urines were processed within 1 to 2 hours after voiding and were kept for this period of time at 4°C. Ten ml of the fresh urine to which penicillin (1,000 U/ml) and streptomycin (1,000 μg/ml) had been added were spun at 2,500 rpm for 15 minutes. The sediment was resuspended into 2 ml of the urine and inoculated in 0.2 ml amounts into 8 culture tubes from which the medium had been drained. After 2 hours adsorption at

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37°C, 2 ml of maintenance medium was placed into each tube. All cultures were thereafter incubated in stationary racks at 37°C. The maintenance medium was changed 24 hours after inoculation and thereafter twice a week, after microscopic examination. Inoculated and control cultures were maintained and observed for 45 to 60 days. Aliquots of urines not utilized for test on HEL cells were frozen and kept at —20°C for further use on HeLa cells. Thawed specimens were inoculated in 0.2 ml amounts into each of 4 HeLa cell culture tubes, which were then observed for 15 days. Throat swabs and stools from leukemic patients and their family contacts were tested for enteroviruses on monkey kidney cells.

Cytomegalovirus isolation and identification. HEL cultures exhibiting advanced cytomegalovirus type cytopathic changes were trypsinized and the cells were mixed with freshly trypsinized normal HEL cells and seeded in tubes or bottles. This usually facilitated the spread of infected cells, and the cytopathic changes in the newly seeded cultures progressed much more rapidly than in the original culture. This was the method regularly used to maintain the isolates in serial passage.

Infected cells were used as virus stocks. They were suspended in Eagle's medium with 20% calf serum and 10% dimethylsulfoxide (DMSO) or 15% glycerol, and frozen in a viable state, in screwcap vials in liquid nitrogen at —195°C. When needed, cells were thawed and used as virus seed.

The cytomegaloviruses isolated were identified mainly on the basis of the characteristic cytopathic effect on HEL fibroblasts, typical intranuclear inclusions in infected cells stained with hematoxylin and eosin, and passage by transfer of cell-free material(11). Varicella virus produces similar changes on HEL cells but it cannot be propagated in a cell-free state(12).

Electron microscopic examinations of several isolates were carried out(13); these revealed herpes-like DNA virus particles 110 mμ in size and containing 162 capsomeres. In immunofluorescence tests all of the isolates tested gave a positive reaction with a human pooled serum of high cytomegalovirus antibody titer but were non-reactive with anti-herpes simplex rabbit serum(11). Recently we have been able to produce specific immune sera in monkeys with several of the isolates and to detect specific neutralizing antibodies by means of a plaque reduction test(14). Sera prepared against several of the isolates appear to neutralize the homologous strain as readily as the Davis strain (kindly supplied by Dr. T. Weller).

Material studied. A group of 101 children ranging in age from several months to 15 years were studied during a period of 2 years (1961-1963). Of these, 72 were patients either hospitalized or followed in the Hematology Clinic. Forty-two of the 72 patients were children with acute leukemia and the remaining 30 children suffered from other diseases: 9 with malignancies other than leukemia, 8 with hemolytic anemia and/or thrombocytopenia, 10 with miscellaneous blood dyscrasias and 3 with nonhematological diseases. A third group of 29 apparently normal subjects, all children of 14 laboratory workers not involved in cytomegalovirus work, was used as a control for the first two groups. In addition to the above 101 children, 4 patients with cytomegalic inclusion disease were also studied.

Results. A total of 666 urine specimens were obtained and processed from the children in the main 3 groups studied. Of these, 51 (8%) were toxic to the fibroblast cultures and could not be followed further. Cultures inoculated with 100 (15%) additional specimens became contaminated within 24 hours after inoculation, apparently as a result of heavy bacterial or yeast content of the urines, and they also could not be followed further. The results obtained with the remaining 515 samples are presented in Table I. Of those, 317 came from the leukemic children, 113 from the children with other diseases and 85 from the control children. Thirty-nine cytomegalovirus isolations were made—18 from 7 of the 42 children with leukemia, 12 from 5 of the 30 children with other diseases and 9 from 6 of the 29

TABLE I. Cytomegalovirus Isolations from Urines of Children in Human Embryo Lung Fibroblast Cells.

Diagnosis	Children	Urine samples			
	No. pos/No. tested	Total pos/Total tested	No. pos/No. tested and observed for: <10 Days	10-20 Days	21-60 Days
Acute leukemia	7/42	18/317	2/45	6/43	10/229
Other diseases	5/30	12/113	0/14	6/27	6/72
Normal controls	6/29	9/85	1/5	5/16	3/64
Total	18/101	39/515	3/64	17/86	19/365
Cytomegalic inclusion disease	4/4	5/7	2/3	3/4	—

TABLE II. Age Distribution of Children in Study.

Age	No. of positive children/No. of children tested in the following groups: Acute leukemia	Other diseases	Normal controls	Total	Cytomegalic inclusion disease
<24 mo	0/2	0/4	0	0/6	4/4
25–36 "	0/3	3/6	2/2	5/11	
37–47 "	1/7	0/2	1/2	2/11	
4– 5 yr	2/15	0/3	2/8	4/26	
6– 7 "	2/7	2/7	1/7	5/21	
8–10 "	2/7	0/3	0/9	2/19	
11–15 "	0/1	0/5	0/1	0/7	
Total	7/42	5/30	6/29	18/101	4/4

control children. In addition 5 of the 7 urine samples tested from the 4 children with cytomegalic inclusion disease were positive.

The first signs of cytopathic changes in the inoculated cultures usually appeared between the 15th and 20th day after inoculation of the urine samples. However, even though 64 specimens were tested and observed for less than 10 days, they yielded 3 cytomegalovirus isolates during this short period (Table I). Most of the specimens (last column of Table I) were tested and observed much longer (21-60 days), as there were occasional specimens which exhibited cytopathic changes only after 20-30 days.

It is evident from the data that there was no difference in number of positive children or number of isolations between the first 3 groups of children. The majority of these children were in the age range of 2 to 10 years (Table II). The virus excretors among the leukemic children tended to be older than those among the "normal" control children (Tables II and III). Four of the 7 positive leukemic children were over 5 years of age while 5 of the 6 control children were under this age. One of the 2 positive control children in the age group of 4-5 years, child BC-5, was the sibling of a 2 year old boy, BC-19, who was also excreting the virus (Table III). The one 7 year old positive child in the control group, BC-14, was a boy who had a history of hemophilia, although we made no attempts to verify the diagnosis (Tables II and III). Except for the 2 siblings, BC-5 and BC-19, each of the remaining positive children in the control group was the only child tested within a family.

Of the 5 positive children in the "other disease" group, 3 were 2-5 years old. One, C-106, eventually expired with thrombotic thrombocytopenia; the second, C-108, had chronic thrombocytopenia and pseudo-hemophilia; and the third, C-132, had Hodgkins disease. Child $C\text{-}106_c$, a 6.5 year old girl, the sister of child C-106, also died with thrombotic thrombocytopenia. The last child in this group, $C\text{-}108_c$, was a 7 year old apparently healthy sister of patient C-108. The only specimen taken from this girl at the time she visited the clinic during her sister's

stay in the hospital yielded the virus. Thus, except for the latter child, the positive children in this group were no different from those with leukemia, in that all suffered from prolonged illnesses and had received steroid treatment at some time.

All 4 children with cytomegalic inclusion disease (Tables II and III) were young children under 2 years of age and all had cytomegaloviruria.

At the initial stages of this study, attempts were made to study siblings and parents of the children with acute leukemia. However, the specimens were collected by the mothers at home and the lapse of time between urine collection and testing proved difficult to control. On the other hand, collection and delivery to the laboratory of urines from the diseased children and the children of the laboratory workers was done in a controlled

TABLE III. Children Excreting Cytomegalovirus in the Urine.

Child No.	Diagnosis	Age, yr	Specimens, No. pos/No. tested	Dates viruses isolated		
				1961	1962	1963
L- 3	Acute leukemia	4	4/18	Nov. 30	Nov. 5	Jan. 4, 11
L- 5	" "	3	1/12	Dec. 12		
L- 6	" "	6	2/8		Sept. 24, Oct. 11	
L-23	" "	5	2/2			Mar. 21, June 20
L-29	" "	10	1/4		Nov. 14	
L-35	" "	9	5/15		Aug. 9, 14, 17, Sept. 6	Jan. 31
L-38*	" "	6.5	3/21			Mar. 30, Apr. 17, May 7
C-106	Thrombotic thrombocytopenia	2.5	2/14			Jan. 4, 15
C-106$_e$	*Idem*	6.5	3/4		Nov. 20, 27, Dec. 18	
C-108	Thrombocytopenia	2.5	4/7			Feb. 5, Mar. 19, May 7, July 2
C-108$_e$	Healthy sibling of C-108	7	1/1			Mar. 15
C-132	Hodgkin's disease	2.5	2/2			Mar. 21, Apr. 4
BC- 5	No disease	5	1/3			June 6
BC- 9	"	3	2/3			May 16, June 5
BC-13	"	4	2/4			June 3, 20
BC-14†	"	7	1/4			May 8
BC-18	"	2	1/3			June 4
BC-19	"	2	2/3			May 20, June 6
C-137	Cytomegalic inclusion dis.	18 mo	1/1			Feb. 15
J.J.	*Idem*	23 "	2/3			July 18, 19
G.H.	"	2 "	1/1	Sept. 30		
F.J.	"	1 wk	1/2			Dec. 10

* Cytomegalovirus was also isolated from a throat swab specimen of a six month old sibling of child L-38 on Apr. 17, 1963.

† Unconfirmed history of hemophilia.

TABLE IV. Frequency of Cytomegalovirus Isolation as Related to Number of Urine Specimens Tested per Child.

No. of specimens tested per child	Total No. of children tested	No. of children with viruria	No. of children negative	% Children with viruria
1	25	1	24	4
2	15	2	13	13
3	23	4	19	17
4 or more	38	11	27	29
Total	101*	18	83	18

* Includes 42 children with leukemia, 30 children with other diseases and 29 normal control children.

manner. Twenty-five siblings of 19 children with leukemia as well as 30 of their parents were tested, all yielding negative results with one exception. A 6 month old baby sister of leukemic patient L-38 (Table III) yielded cytomegalovirus in a throat swab taken at the time of a visit to the clinic and tested on HEL cells. No urines were taken from this baby and no systematic efforts were made to isolate cytomegalovirus from mouth or throat swabs of any of the patients or their family contacts.

Most of the positive children excreted the virus repeatedly (Table III). Some of the children, as leukemic child L-3, excreted the virus intermittently for over a year (Nov. 30, 1961-Jan. 11, 1963).

The majority of the children with viruria were children sampled more than once. An attempt was made (Table IV) to correlate frequency of virus isolation with number of urine specimens tested per child. Since the proportions of children with viruria were the same among the leukemic patients, the children with other diseases and the control children, they were analyzed together. Twenty-five children were sampled only once, 15 twice, 23 three times and 38 four or more times. The percent of positive children increased with the increase in number of urines tested per child. Only 1 (4%) of the 25 children sampled once was found to excrete the virus while 29% (11 of 38) of the children tested 4 or more times were positive. This was true for the children with leukemia or other diseases as well as for the control children, for they were evenly distributed as to frequency of sampling. As expected, all 4 of the children with cytomegalic inclusion disease excreted the virus even though 2 were tested only once (Table III).

The cytomegalovirus isolations during this 2 year study period were evenly distributed through the year with no seasonal predilection.

Cytomegalovirus was the only virus we isolated from urines during this period with one exception. Adenovirus type 3 was isolated on HEL fibroblasts repeatedly from urine samples of a 4 year old child with hypoplastic anemia. Eight of the 12 urine samples taken between Dec. 1962 and April 1963 yielded the virus. (The child has not been followed further.) Four of the 8 samples positive for adenovirus in HEL cultures were also positive for this agent when tested on HeLa cells, after the urines had been kept frozen at —20°C. Human embryonic kidney or HeLa cells were much more suitable for the adenovirus passage and titration—the TCD_{50} titers in these cells were usually 3-4 $\log_{10}$ higher than those obtained in HEL cells. However, the latter cells were susceptible for primary isolation, although the cytopathic changes appeared and progressed more slowly than on epithelial cells.

Viral isolations in HeLa cells were attempted with many of the urine samples kept frozen at —20°C. Negative results were obtained with 120 urines from 21 leukemic children, with 34 samples from 10 of their siblings and with 51 samples from 12 of their parents. Except for the single patient already described, 44 urine samples from 19 children with diseases other than leukemia were also negative. The same negative results were obtained with 81 urines from the 29 normal control children.

Work is now under way to compare antigenically some of the cytomegalovirus strains isolated. Employing a plaque reduction neutralization test on HEL fibroblasts we have been able to measure satisfactory homologous titers (1:32 → 1:256) in sera of monkeys conventionally immunized with several of our isolates as well as with the Davis and Kerr strains obtained from Dr. T. Weller(14). Cross neutralization tests are now underway to further analyze representative isolates.

Discussion. The initial aim of this work was to elucidate the spectrum of known viruses harbored by children with acute leukemia and by their family members. Since increasing numbers of viruses have been isolated from urines(2,3,6,15-18), we utilized urines in addition to throat swabs and stools for isolation purposes. Children coming to the Hematology Clinic with diseases other than leukemia were to be used as controls. When it became apparent that a significant number of these children had too many clinical and laboratory aspects in common with the leukemic children, a third group of "normal" control children was added to the study.

Except for a 4 year old child with hypoplastic anemia who repeatedly excreted adenovirus type 3 in its urine, cytomegalovirus was the only agent isolated from the urines of the other children in the study.

The data obtained in this study demonstrate that there was no difference in rate of urinary excretion between the leukemic children or other chronically ill children and "normal" children of comparable age in the same community. While Hanshaw and Weller (3) demonstrated viruria in 4 of 50 children with various malignancies, in another study (7) they failed to demonstrate viruria in 136 hospitalized children free of cytomegalic inclusion disease. The reason for the discrepancy between the findings of these workers and ours may very well lie in the age distribution of the children studied. While the children with malignant diseases studied by those authors(3) were older children up to 15 years of age, the 136 other hospitalized patients(7) were very young children (27 newborns, 86 children under one year and the remainder under the age of two). Most of the children studied by us were over 2 years of age except for the children with cytomegalic inclusion disease. Rowe and coworkers(6) have reported a high incidence of cytomegalovirus isolation among institutionalized children 8 months to 4 years old, and assumed that this was due to the extremely close contact between the children within this group. However, we also found a high rate of cytomegaloviruria among normal children from separate households. Even if one excludes child BC-14, who had a history of hemophilia, and child BC-5, who was apparently healthy but was a family contact of positive child BC-19, 15% (4 of 27) of the remaining healthy children excreted cytomegalovirus in their urine. This rate was not much different from the 17% (7 of 42) positive leukemic children or from the 11% (3 of 28, after subtracting children $C\text{-}106_c$ and $C\text{-}108_c$ as siblings of positive children) of the children with diseases other than acute leukemia. Our findings, together with the failure of others(6,7,8) to isolate the virus from infants other than patients with cytomegalic inclusion disease, substantiate the findings of Rowe *et al*(19). They demonstrated a high rate of cytomegalovirus complement fixing antibodies in newborns (71%), very low rates (14%) in children between 6 months and 2 years of age and gradual acquisition of antibodies after the age of 2, reaching a peak at 35 years with a rate of 81% positives after that age.

The high rate of cytomegaloviruria in our study could be attributed also to the multiple urine samples tested from most of the children. Indeed, as illustrated in Tables III and IV, 15 of the 18 positive children (excluding the children with cytomegalic inclusion disease) were sampled 3 or more times.

As regards enterovirus excretion, the leukemic patients studied in this laboratory did not reveal any demonstrable deviation from the rates expected for children of this age in a community. Some enteroviruses were isolated on rhesus monkey kidney cells from either throat swabs or stool suspensions from

the leukemic children as well as from their family contacts. Most of the isolates were the polio type 1, 2, and 3 strains with which the community was orally vaccinated during the time of specimen collection. In addition coxsackievirus A9 was isolated from a leukemic child and from one of his siblings and an echovirus type 7 from another leukemic child. No difference was found in rate of poliovirus excretion between the leukemic children and their siblings. Usually the same virus type was isolated from the sick child and the siblings within a family and occasionally from one of their parents.

Summary. Virus isolations were attempted during a 2 year yeriod (1961-1963) from urine samples of 101 children: 42 with acute leukemia, 30 with diseases other than leukemia and 29 healthy children, most of them 2 to 10 years old. Thirty-nine cytomegalovirus isolations were made—18 from 7 of the 42 children with acute leukemia, 12 from 6 of 30 children in the "other disease" group and 9 from 6 of the 29 control children. In addition 5 isolates were obtained from 7 urines of 4 infants with cytomegalic inclusion disease. Adenovirus type 3, the only other virus found in the urine samples, was isolated from 8 of 12 specimens collected over a 5 month period from a child with hypoplastic anemia. Enterovirus recoveries from the throat and stools of the leukemic patients yielded normal patterns for children of the age group studied. Coxsackievirus A9, echovirus 7, and polioviruses 1, 2, and 3 were isolated; the polioviruses were found with high frequency as oral poliovaccines were widely distributed in the community during this study.

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Failure of Integrated Cardiac Action at Supernormal Heart Rates.* (29655)

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It is well known that as the heart rate increases, under intrinsic or imposed drive, processes within the heart accelerate. Refractory periods shorten, electrical and mechani-

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