THE JOURNAL OF INFECTIOUS DISEASES • VOL. 142, NO. 1 • JULY 1980

Microneutralization of Cytomegalovirus

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A simple microneutralization test for cytomegalovirus (CMV) is presented. Using a laboratory adapted stock virus, definite results were obtained after 10 days. All patients with primary CMV infection showed an antibody rise and/or seroconversion; however, neutralizing antibody appeared only seven weeks after the onset of clinical symptoms. In control patients without evidence of recent primary infection, there was a complete concordance of the presence of complement-fixing and neutralizing antibodies.

Neutralization tests [1–5] with cytomegalovirus (CMV) have not been very popular because CMV is a slow-growing virus and not easy to obtain as high titered, cell-free virus. However, the determination of neutralizing antibodies may be more significant in clinical situations than, for example, levels of CF antibodies. This situation is especially true when we seek to evaluate the responses of individuals to natural infection or CMV vaccines. Furthermore, neutralization tests, as has been shown for herpes simplex viruses (HSV) [6, 7], can permit us to define and identify viral subtypes. This paper describes the adoption to CMV of a simple microneutralization test [7] which has proven its value for typing HSV isolates and antisera.

Materials and Methods

Cells. Diploid human fibroblast cells derived from newborn foreskin (FS-9; initiated at the Center of Disease Control, Atlanta, Ga.) were used. These cells were propagated as described previously [7].

Virus. CMV (strain 268a) was originally isolated from the urine of a kidney transplant recipient. The original isolate was characterized as CMV by its focal, slowly progressing CPE, with eosinophilic intranuclear and rare cytoplasmic inclusions as well as mononuclear giant cells after staining with hematoxylin and eosin. The virus was identified as CMV by the immunofluorescence technique using a highly specific human antiserum (performed by Dr. U. Krech, Institut für Medizinische Mikrobiologie, St. Gall, Switzerland). Furthermore, specific rabbit antisera containing a high titer of antibody to HSV type 1 (HSV-1) and HSV type 2 (HSV-2) did not neutralize the agent (see below).

The original isolate was passaged 16 times by cell scraping. After the 16th passage, the cell monolayer was trypsinized (0.04% trypsin and 0.54 mM EDTA) when ~80% of the cells showed CPE. After centrifugation at 120 *g* for 10 min, the cells were resuspended in distilled water and sonicated (Sonifer B-12; Branson Sonic Power Company, New Haven, Conn.) at a force of 2 for 22 sec. Microscopic examination showed almost complete cell disruption.

This suspension was clarified at 1,000 *g* for 15 min, and sorbitol was added to a final concentration of 10%. The virus was aliquoted into ampules and stored at −80 C. From this pool one ampule was added to a 500-ml roller bottle containing a complete cell monolayer. When ~80% of the cells showed CPE, the culture was trypsinized, and the cells were added to confluent monolayer cultures in four 2,000-ml Roux bottles (Müller and Krempel, Carouge, Switzerland). When ~80% of the cells showed CPE, the cultures were trypsinized and sonicated, and the clarified supernatant was stored in 10% sorbitol at −80 C. This virus was used for all experiments.

Microtitration and microneutralization technique. A procedure similar to that described for HSV was used [7]. For titration of virus, 10-fold dilutions of virus were prepared in growth medium (Dulbecco's medium, supplemented with bicarbonate and antibiotics [7] and 10% heat-inactivated [56 C for 30 min] fetal calf serum). Then 0.05 ml of each dilution of virus was placed in each of six wells of sterile, disposable microtest culture plates with 96 flat-bottom wells (M29ART, Microtiter system; Cooke Engineer-

Received for publication April 30, 1979, and in revised form January 29, 1980.

This work was partially supported by a research grant from the Swiss Cancer League.

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ing, Alexandria, Va.). Immediately, 0.05 ml of a cell suspension containing 200,000 cells/ml in growth medium was added. The plates were covered and incubated at 36 C in a moist atmosphere of 5% CO_2 in air. The CPE was read using an inverted microscope. All wells were scored as + (CPE present) or − (no CPE), and end-point dilution titers were calculated using the Kärber method [8].

For serum neutralization studies, twofold dilutions were prepared in growth medium. First, 0.025 ml of each dilution was placed in three replicate wells. Then, 0.025 ml of viral suspension containing ~200 $TCID_{50}$/0.05 ml was added to two of the three wells. The remaining well received 0.025 ml of growth medium (serum control). Then, 0.025 ml of a 1:4 or 1:8 dilution of either fresh or heat-inactivated (56 C for 30 min) guinea pig serum (stored at −80 C) was added to all of the wells. The plates were then incubated at different temperatures for various intervals (see Results). Finally, 0.05 ml of the cell suspension was added, and the plates were incubated in the CO_2 incubator for various intervals (see Results).

When plates were read at 10 days, a change of medium was not necessary. The results were read as described above, and serum titers were expressed as the reciprocals of the highest dilution of serum that inhibited viral CPE.

CF tests [9] and determination of CMV-specific IgM antibody [10] were performed by Dr. U. Krech using the Davis strain of CMV as the antigen.

Results

Titration of virus. Viral titers were between log 2.66 and log 3.83 $TCID_{50}$/0.05 ml. Exposure of the diluted virus to different temperatures before addition of cells appeared to modify the course of infection. When the virus was held for 30–120 min at 4 C before it was placed into the wells of the microtest plates, the subsequent development of CPE was delayed, so that at 10 days, the virus held at 4 C appeared to have a titer 1 log lower than the same virus held at 22 C or 37 C. However, CPE eventually developed so that after 18 days of incubation, the final titers were comparable. Addition of either fresh or heat-inactivated guinea pig serum did not change the kinetics of CPE development or the final titer of virus.

Serum neutralization. No consistent difference of neutralizing titer was observed when mixing periods of virus and serum were varied (30, 60, and 120 min) or when mixing was done at different temperatures (4, 22, and 37 C). Therefore mixing was done at 37 C for 30 min in all further experiments. Since reading the test after 15, rather 10, days of incubation changed in titers very little (only, and that inconstantly, about half a dilution lower at 15 days), all tests were read at 10 days. Fresh guinea pig serum increased the titers up to 11-fold (mean, fourfold) (table 1). Several sera were positive only after addition of fresh guinea pig serum.

Patient sera. Sequential sera from 11 patients who appeared on clinical and virologic grounds to have primary CMV infection were examined. CMV was isolated from all except one patient, and all developed either CMV-specific fluorescent IgM antibody or a fourfold rise in CF antibody titer (tests performed by Dr. U. Krech). No serum contained detectable neutralizing antibodies earlier than seven weeks after the beginning of the illness. After 10 weeks, however, multiple sera from all patients were positive. Seroconversion was documented in eight patients (table 1 and figure 1).

Ten sera from unpaid blood donors and 10 sera containing CF antibodies to HSV from patients from whom HSV was isolated were tested. Five of each group had positive titers of neutralizing antibody to CMV. Their values were compared with titers of CF antibody to CMV. No serum that lacked neutralizing antibody gave a positive CF test and vice versa.

Discussion

Using the microneutralization technique, we demonstrated the appearance of neutralizing antibodies in the sera of all patients suspected of having primary CMV infection, when tested 10 weeks or later after the onset of clinical symptoms. However, none was positive before seven weeks. Late appearance of neutralizing, in contrast to CF or fluorescent, antibodies has already been noted by Spencer and Andersen [11]. Although not tested in our laboratory, it is possible that early IgM antibodies have little neutralizing activity [12]. The clinical implication of this is not clear, but it is interesting to note that the clinical course as well as viral excretion in CMV infections is protracted. In our test system, addition of fresh guinea pig serum enhanced neutralizing antibody

Table 1. Enhancement of serum neutralization titers by addition of fresh guinea pig serum in patients with clinically suspected primary infection with cytomegalovirus (CMV).

Patient no.	Weeks after beginning of illness	Reciprocal titer of antibody		Reciprocal neutralization titer with guinea pig serum	
		CMV-specific IgM	CF	Fresh	Heat-inactivated
1	1	ND	<10	ND	ND
	4	320	40	<5	<5
	15	ND	ND	10	<5
2	2	640	80	ND	ND
	5	320	240	<5	<5
	16	20	240	120	10
	57	ND	ND	30	10
3	9	80	240	10	<5
	58	ND	ND	160	20
4	3	320	30	ND	ND
	4	ND	120	<5	<5
	23	20	ND	20	10
5	1	5	<10	<5	<5
	9	20	240	5	<5
6	5	80	⩾160	<5	<5
	23	ND	ND	20	15
	76	<5	⩾160	40	15
	90	ND	120	20	10
7	13	40	⩾160	20	10
	16	10	⩾160	15	<5
	30	ND	ND	15	<5
8	2	80	240	<5	<5
	6	40	240	<5	<5
9	3	ND	ND	<5	<5
	8	80	240	<5	<5
	25	<5	240	15	<5
10	12	20	128	ND	15
	58	ND	ND	120	60
11	2	ND	10	<5	<5
	7	ND	⩾160	10	<5

NOTE. ND = not done.

titers about fourfold, presumably due to the action of complement. Several sera became positive only after addition of fresh guinea pig serum. Its addition is therefore mandatory when this technique is used.

Complement enhancement is not unique to neutralization of CMV but has also been noted with other viruses, especially those of the herpes group [13, 14]. However, complement enhancement of human sera has been found irregularly in CMV plaque-neutralization assays [5, 12, 15]. This observation is in contrast to those with animal sera, some of which are completely dependent on complement for neutralizing activity [3, 12]. There was a complete correlation between the microneutralization test and the CF test in twenty control patients. No patient with a negative titer in one test had a positive one in the other.

This microneutralization technique [7] has several advantages over other neutralization tests; for example, transfer of virus-serum mixtures is avoided because the cells are added to the same well. Owing to the microtechnique, only small amounts of serum (0.025 ml for each serum dilution), of virus, and of cells are necessary. It is therefore easy to test multiple sera in the same

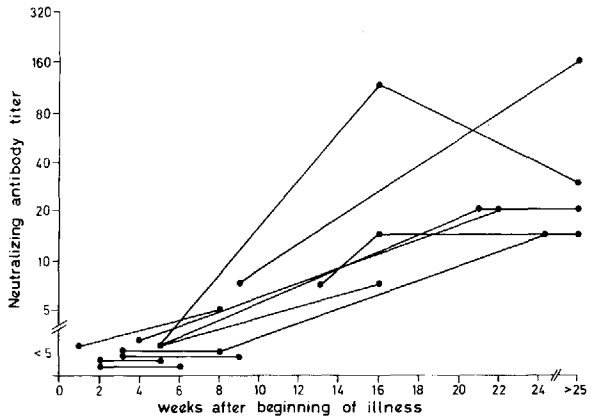

Figure 1. Development of neutralizing antibodies in patients with suspected primary cytomegalovirus infection.

test, and furthermore, the test can be performed in multiple duplicates, eventually permitting subtyping of CMV isolates [16]. Indeed, a similar test was originally used to detect subtypes of HSV isolates [6]. Finally, the test can be performed at room temperature or at 37 C. The peculiar effect of preincubation of dilutions of virus at 4 C on reducing the subsequent development of CPE is unexplained. While it may simply be some reversible physical phenomenon such as aggregation, it may also be related to the resistance of CMV to elevated temperatures which has been noted by Vonka and Benyesh-Melnick [17].

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