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Establishment of Titration System for Human Herpesvirus 6 and Evaluation of Neutralizing Antibody Response to the Virus

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The susceptibilities of seven T-cell lines to human herpesvirus 6 (HHV-6) infection were examined. MT-4 cells were the most susceptible of these lines to infection with this virus. Therefore, chemically adhered MT-4 cell monolayers were used for infectious HHV-6 assay by indirect immunofluorescent-antibody (IFA) staining. When cell monolayers were fixed 30 to 45 h postinfection, the foci stained with IFA were easy to count and a linear relationship was observed between the number of foci and the virus concentration. MT-4 cell monolayers were also used for a focus reduction neutralizing-antibody test. In this test, sera from patients in the convalescent stage of exanthem subitum all showed significant neutralizing activity (1:80 to 1:320), whereas sera from patients in the acute stage of disease showed no detectable neutralizing activity. The titers of neutralizing antibody correlated well with the levels of anti-HHV-6 antibodies detected by IFA.

The isolation of a new human herpesvirus from patients with lymphoproliferative disorders and acquired immune deficiency syndrome and from normal adults was recently reported by several groups (2, 3, 7, 13, 14). This virus differs from other human herpesviruses in antigenicity, in the structure of its DNA, and in its unique cell tropism (5, 13) and so was named human herpesvirus 6 (HHV-6) (1, 7, 9). Recently, we reported that HHV-6 causes exanthem subitum (15). This virus is reported to have a high affinity for T lymphocytes (1–3, 7–9), and because of this unique cell tropism, the 50% tissue culture infective dose ($TCID_{50}$) in cord blood mononuclear cells has usually been used for quantitation of viral infectivity. However, measurement of the $TCID_{50}$ is time-consuming, requires large numbers of cells, and is not suitable for accurate measurement of the neutralizing (NT)-antibody titer. Here, we describe a convenient method for assay of this virus and an NT test using MT-4 cells and indirect immunofluorescent-antibody (IFA) staining.

MATERIALS AND METHODS

Virus preparation. Cord blood mononuclear cells infected with the HHV-6 HST strain, which was isolated from an exanthem subitum patient (15), were precipitated by centrifugation and suspended at 5×10^6 cells per ml in phosphate-buffered saline with 0.2% gelatin and 5% sucrose. This suspension was sonicated for 30 s and centrifuged at 2,000 × *g* for 20 min, and the supernatant was stored at −70°C until use.

Cells. Seven T-cell lines, MT-4 (kindly supplied by K. Ikuta in our Institute [11]) and HPB-ALL, Molt-3, Molt-4, CCRF-CEM, HUT-78, and H9 (kindly supplied by J. Minowada, Hayashibara Institute [10]), were maintained at 37°C in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), L-glutamine, penicillin, and kanamycin. Cord blood mononuclear cells were cultured at 37°C in RPMI 1640 medium supplemented with 10% FCS, L-glutamine, penicillin, and kanamycin, 0.1 U of recombinant human interleukin-2 per ml (Takeda Chemical Industries), and 5 μg of phytohemagglutin per ml (Honen Oil Co.).

Infection of T-cell lines with HHV-6. The seven T-cell lines described above were infected with HHV-6 at a multiplicity of infection of 0.1. The pellet of 5×10^6 viable cells was mixed with 0.5 ml of virus preparation and incubated for 2 h at 37°C for adsorption of the virus. Then the infected cells were washed once and suspended at 5×10^5 cells per ml in RPMI 1640 medium containing 10% FCS. The virus-infected cells were put into a 24-well plastic tray (1 ml per well) and incubated at 37°C in a humidified CO_2 incubator.

Every day until 7 days after infection, samples of the cells were smeared on microscope slides and fixed. HHV-6-specific antigens were stained by an IFA method in which sera from convalescent exanthem subitum patients and fluorescein isothiocyanate-labeled goat antibodies to human immunoglobulin G (Cooper Biomedical, Inc.) were used. Antigen-positive cells were examined under a fluorescence microscope. For determination of the percentage of antigen-positive cells, over 500 cells were counted.

On various days after infection, T-cell suspensions from the wells were transferred to plastic tubes and centrifuged. The supernatant fluids were stored at −70°C for titration of extracellular virus, while the cell pellets were suspended in 1 ml of phosphate-buffered saline with 0.2% gelatin and 5% sucrose, sonicated, and centrifuged, and the supernatants were stored at −70°C for titration of intracellular virus. These samples of intracellular and extracellular virus were examined for virus infectivity by $TCID_{50}$ assay.

Preparation of cell monolayers. Cell monolayers were prepared on spot glass slides (heavy Teflon coating slide, 24 well; Bokusui Brown) coated with poly-L-lysine (molecular weight 70,000 to 150,000; Sigma Chemical Co.) which was solubilized in phosphate-buffered saline. Volumes of 25 μl of poly-L-lysine (100 μg/ml) were placed on each spot on the slides. The slides were then incubated for 120 min at room temperature and rinsed three times with phosphate-buffered saline. MT-4 cells were washed three times and suspended in RPMI 1640 medium without FCS (2.0×10^6/ml). Volumes (25 μl) of cells were applied to each poly-L-lysine-coated spot and incubated for 40 min at 4°C. Then unbound cells

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were removed by being washed three times with RPMI 1640 medium without FCS.

Assay of virus infectivity with MT-4 monolayers. Serial dilutions of stock virus were inoculated onto monolayers on spot slides (20 μl per spot). After an adsorption period of 120 min at 4°C, the virus suspension was removed by aspiration, and RPMI 1640 medium supplemented with 2% FCS was added to the monolayers. The spot slides were kept in a moisture chamber and incubated at 37°C in a CO_2 incubator for various periods (25 to 60 h), and the cells were then fixed and stained by the IFA technique. For the IFA technique, we used mouse monoclonal antibodies (T. Okuno, H. Shao, H. Asada, K. Shiraki, M. Takahashi, and K. Yamanishi, submitted for publication) which react to HHV-6 antigen or convalescent-phase sera from patients with exanthem subitum. Antibody titers of these sera were 1:20,000 and 1:2,560 by IFA test, and antibodies were diluted 1:100 and 1:50. Fluorescein isothiocyanate-labeled goat antibodies to mouse immunoglobulin G or to human immunoglobulin G (Cooper Biomedical) were also used as the secondary antibody.

$TCID_{50}$ assay. Suspensions (100-μl volumes) of MT-4 cells or cord blood mononuclear cells at 5×10^5 cells per ml were plated into flat wells of microdilution plates (Corning Laboratory Sciences Co.). The same volume of 10-fold-diluted supernatant was then added to each well. Quadruplicate experiments were performed at each dilution. The plates were incubated at 37°C for 10 days, and half of the medium (RPMI 1640 medium with 10% FCS and antibodies) was changed every 4 days. Virus antigens were checked by the IFA method, and the 50% endpoint was calculated by the method of Reed and Muench (12).

NT test. MT-4 monolayers on spot slides were also used for NT tests. Sera were heated at 56°C for 30 min before use. Serial twofold dilutions of serum were mixed with an equal volume of HHV-6 suspension (50 focus-forming units/10 μl) and incubated at 37°C for 1 h. Duplicate monolayers of MT-4 cells were inoculated with 20 μl of each mixture and incubated for 120 min at 4°C. The inocula were then removed, and the cells were washed and covered with RPMI 1640 medium containing 2% FCS. The cultures were incubated for 35 h at 37°C in a CO_2 incubator, fixed, and stained by the IFA method.

RESULTS

Infection of T-cell lines with HHV-6. To find a suitable cell line for use in assay of infectious virus, we examined the susceptibilities of seven T-cell lines (MT-4, HPB-ALL, Molt-3, Molt-4, CCRF-CEM, HUT-78, and H9) to infection with cell-free HHV-6. MT-4 cells were found to be the most susceptible to virus infection, followed by HPB-ALL cells (Fig. 1A). Other cell lines were much less susceptible, with less than 5% of the cells being fluorescent for up to 7 days after inoculation (data not shown). Furthermore, infected MT-4 cells were more clearly distinguishable than HPB-ALL cells because MT-4 cells in general are larger than HPB-ALL cells. Intracellular virus released by sonication of MT-4 cells infected with HHV-6 was detected on day 3 postinoculation, and the virus titers at this time were more than 10^3 $TCID_{50}$ per ml (Fig. 1B). In contrast, virus released into the supernatant fluids of infected MT-4 cells (extracellular virus) was not detected until day 4 postinoculation, and the titers were about 1/100 of those of intracellular virus (Fig. 1B).

Virus infectivity assay with MT-4 monolayers. The infectivity of stock virus to chemically adhered MT-4 cell monolayers was examined. Virus-infected MT-4 cells stained by IFA could not be distinguished clearly for up to 20 h postinfection. At 25 h after infection, cells infected with HHV-6 could be distinguished and counted, but the fluorescence was not strong. From 30 to 45 h after infection, however, virus antigens were clearly detected by IFA, and the foci were easy to count. At this time, one focus consisted of one or several antigen-positive cells. The number of foci did not change appreciably between 30 and 45 h postinfection (Fig. 2). However, as MT-4 cells gradually aggregated during incubation, they started to become detached from the slide as of 50 h postinfection, so the number of foci started to decrease (Fig. 2). Figure 3 shows the typical appearance of cells stained by the IFA method 35 h after infection. The number of foci was linearly correlated with the virus concentration (Fig. 4). Furthermore, there was a correlation

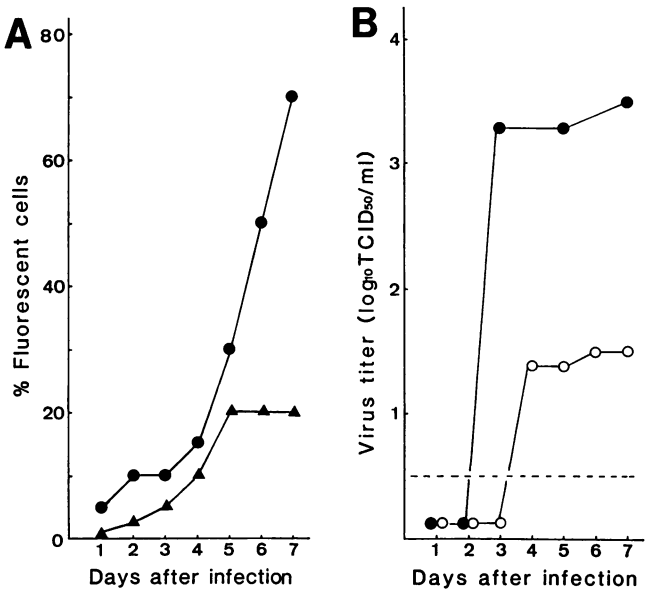

FIG. 1. (A) Kinetics of increase in HHV-6-specific antigen-positive cells detected by the IFA method. The percentages of antigen-positive MT-4 (●) and HPB-ALL (▲) cells were determined by the IFA method every day for 7 days postinfection. (B) Comparison of amounts of intracellular (●) and extracellular (○) virus in HHV-6-infected MT-4 cell cultures. Intracellular and extracellular virus samples were prepared from HHV-6-infected MT-4 cell cultures as described in the text. The virus titers of these samples were determined by $TCID_{50}$ assay in MT-4 cells. The detection limit is shown (– – –). The tests were independently carried out at least three times, and representative results are shown.

TABLE 1. Comparison of virus titers[a]

Virus stock	Virus titer				
	FFU/ml for MT-4	$TCID_{50}$/ml			
		MT-4	CB1	CB2	CB3
1	7.5×10^4	6.3×10^4	2.0×10^3	1.0×10^3	3.2×10^4
2	1.5×10^5	3.2×10^5	3.1×10^3	1.0×10^3	3.2×10^5
3	6.0×10^5	6.3×10^5	3.2×10^4	6.3×10^3	3.2×10^5

[a] Infectivity titers were determined as focus-forming units (FFU) by using chemically adhered MT-4 monolayers, as described in the text, and as $TCID_{50}$ by using MT-4 cells and three samples of human cord blood mononuclear cells (CB1 to CB3).

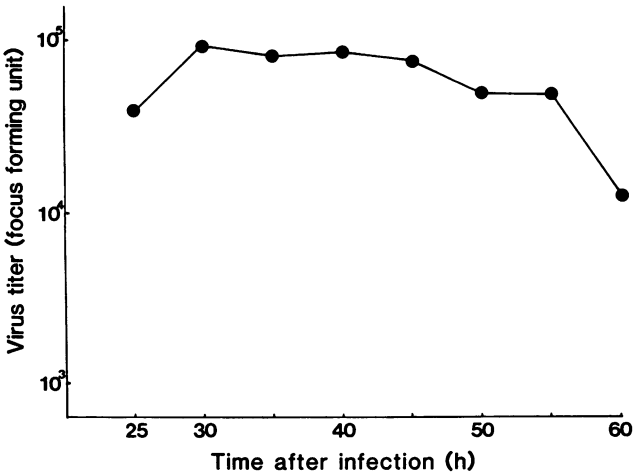

FIG. 2. Effect of incubation period of MT-4 cells infected with HHV-6 on the number of foci. MT-4 cell monolayers were infected with HHV-6. At various times after infection, the cells were fixed and stained by the IFA method. Virus titers were calculated from the numbers of foci. The tests were done three times, and representative results are shown.

between the virus titers determined by our method and the $TCID_{50}$s for MT-4 cells (Table 1). In contrast, variable results were obtained for the virus titers determined with cord blood mononuclear cells.

NT-antibody assay. Sera from five infants in the acute and convalescent stages of exanthem subitum were examined by the NT and IFA tests against HHV-6 (Table 2). Sera obtained in the acute stage showed no significant NT activity (<1:20). In contrast, all sera obtained in the convalescent stage showed high NT activity (1:80 to 1:320), and the activities were essentially proportional to the antibody titers determined by the IFA test.

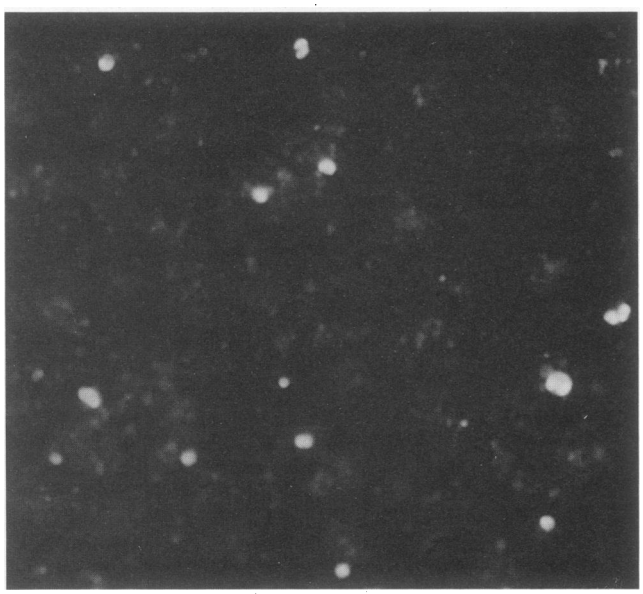

FIG. 3. HHV-6 foci stained by the IFA method. Chemically adhered MT-4 cell monolayers on spot slides were infected with HHV-6. At 35 h postinfection, the cells were fixed and stained by the IFA method.

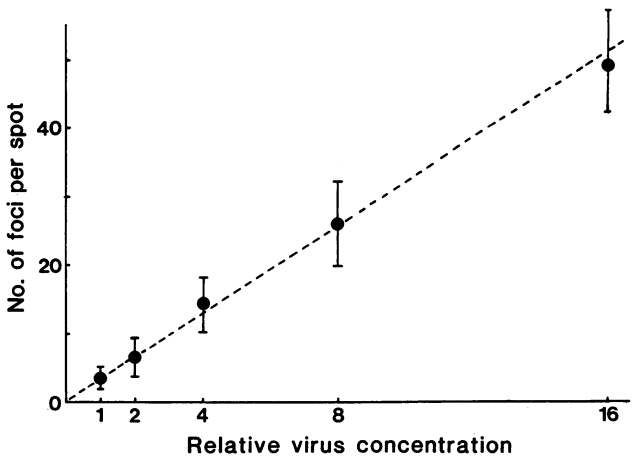

FIG. 4. Linear relationship between the number of foci and virus concentration. MT-4 cell monolayers on spot slides were infected with serially twofold-diluted HHV-6. At 35 h postinfection, the cells were fixed and stained by the IFA method. Triplicate experiments were performed at each dilution.

DISCUSSION

In this work, we first established a new method for quantitation of infectious HHV-6. So far it has seemed difficult to use a plaque or focus formation technique for quantitation of infectious HHV-6, because this virus infects mainly T lymphocytes (1–3, 7–9). However, Harada et al. established a plaque-forming assay with chemically adhered MT-4 cells to determine the number of infectious HIV particles (6). Therefore, we examined the possibility of using chemically adhered cells to assay infectious HHV-6 particles, too.

To determine which cell line was useful for this assay, we first tested the susceptibilities of seven T-cell lines to HHV-6 infection. MT-4 cells were the most susceptible of these cell lines and appeared to be suitable for use in quantitation of this virus (Fig. 1A). Therefore, for infectious virus assays we used chemically adhered MT-4 cell monolayers and IFA staining. Foci were easy to count when the cell monolayers

TABLE 2. NT and IFA tests against HHV-6

Patient	Days after onset	Antibody titer	
		NT[a]	IFA[b]
1	3	<1:20	<1:20
	32	1:160	1:640
2	3	<1:20	<1:20
	33	1:80	1:320
3	1	<1:20	<1:20
	32	1:320	1:640
4	4	<1:20	<1:20
	35	1:160	1:320
5	2	<1:20	<1:20
	33	1:80	1:320

[a] Titers expressed as reciprocals of the highest serum dilutions causing an 80% reduction in focus formation.

[b] Titers expressed as reciprocals of the highest serum dilutions giving specific fluorescence.

were fixed and stained between 30 and 45 h postinfection, and during this period their number was essentially constant. The relationship between the number of foci and the virus concentration represents focus formation, according to the one-particle hypothesis (4). It is reasonable that no secondary foci seemed to be formed in this assay system, because virus released into the supernatant fluid of infected MT-4 cells was not detectable for the first 3 days postinfection (Fig. 1B). Furthermore, the virus titers determined by this new method correlated well with the $TCID_{50}$s in MT-4 (Table 1). The $TCID_{50}$ in human cord blood mononuclear cells has usually been used for quantitation of infectious HHV-6. However, there are several problems with this assay system. For example, it is time-consuming and requires large numbers of cells and relatively large volumes of virus samples. Furthermore, primary mononuclear cells are highly heterogeneous, so results on HHV-6 infectivity are not reproducible (Table 1). Our method has the advantages that it is rapid and requires small samples of cells and virus.

We also applied the focus formation technique described above in an NT test. In this test, sera from five infants with exanthem subitum showed no detectable neutralizing activity in the acute stage of disease but high NT activity in the convalescent stage (Table 2). This result suggests that the NT-antibody titer can be determined accurately with this system. Quantitation of NT antibody seems valuable for evaluating the clinical status of patients infected with HHV-6 who have a lymphoproliferative disorder, acquired immune deficiency syndrome, or some other diseases.

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