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Detection of Human Cytomegalovirus and Analysis of Strain Variation

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I. INTRODUCTION

Human cytomegalovirus (CMV) is increasingly recognized as an important human pathogen (43). This virus produces a series of clinical pictures varying from classic "cytomegalic inclusion disease" to intrauterine death, prematurity, congential defects, infectious mononucleosis, postperfusion syndrome, and interstitial pneumonia in patients who have undergone organ transplantation (29, 43). It is likely that other diseases or syndromes caused by human cytomegalovirus have not yet been recognized. The observations that human CMV may cause normal human fibroblast cells to multiply and grow in soft agarose (28) and that human CMV has the ability to transform hamster-embryo fibroblasts (1) suggest that human CMV might have oncogenic potency comparable to that of herpes simplex, Epstein–Barr virus (EBV), and other herpes-group viruses.

Human CMV has been isolated from saliva, urine, blood, human milk, cervical secretions, various tissue specimens, and even from human semen (30, 43). In congenital and postnatal infections, the virus is excreted from saliva and urine for a sustained period and then apparently enters a latent state. Recurrence of excretion of CMV in pregnancy (31, E. R. Alexander, and our unpublished observations), reactivation of CMV replication in immunosuppressed patients (8), and the presence of infectious CMV in fresh donor bloods (7) strongly indicate the latency of human CMV infections as found with other herpes-group viruses.

The peculiar thermal and chemical lability of human CMV, the slow appearance of cytopathic effects, the slow growth cycle in tissue culture and the remarkable antigenic variation, plus the lack of specific heterologous hyperimmune serum present complicating factors in the diagnosis of human CMV infection. In this communication we would like to discuss some of our new approaches for virus detection, as well as the study of strain differences as approached by nucleic acid hybridization, restriction endonuclease analyses of the CMV genome, and antigenic analyses.

II. DETECTION

Detection of cytomegalovirus infection by isolation in tissue culture and immunologic procedures (e.g., neutralization, fluorescent antibody, and platelet-agglutination tests, complement-fixation, detection of CMV IgM, and immunoperoxidase labeling technique have been described previously in several publications and review articles (3, 6, 14, 16, 17, 22, 27). Here we would like to introduce several new techniques which are applicable to the human cytomegalovirus system.

(A) Anti-Complement (C'3) Immunofluorescence Test (ACIF)

The ACIF test was recently introduced by Reedman and Klein in the Epstein–Barr virus (EBV) system for detection of the EBV-specific nuclear antigen (EBNA) (36); this antigen is difficult or impossible to detect by indirect immunofluorescence. The ACIF test is based on the ability of specific antigen and antibody reactions to fix complement; with FITC conjugated anti-complement serum, the location of the fixed complement can be demonstrated. This test has proven to be more sensitive than the classic direct or indirect immunofluorescence tests (13, 19) due to its amplifying effect and because it detects both IgM and IgG classes of antibody–antigen reactions that fix complement. Anti-viral hyperimmune sera prepared from cattle and many species of birds cannot be used in this test because these sera are inactive in fixing complement.

In brief, frozen sections or exfoliated cells were first fixed with ice-chilled acetone. Anti-CMV hyperimmune serum prepared in guinea pigs (22) was mixed with complement and applied to the samples. These were incubated in a moist CO_2 incubator for 40 min. We used human serum without EBV, herpes simplex, CMV, and varicella–zoster CF antibodies as the complement source. After extensive washing, the FITC-conjugated anti-human C^3 (β_1C/β_1A globulin, Hyland) together with rhodamine counterstain were applied to the sample and incubated for another 45 min. Essential to correct interpretation of this test is a control specimen to which CMV antiserum is not applied. This control will detect the presence of any cells which process complement receptor sites that are able to fix complement and give a false positive result (e.g., B lymphocytes).

Figure 1 shows the detection of CMV antigens by the ACIF test. The specimen is a frozen section of kidney from a congenitally CMV-infected infant. The location of CMV antigens is mainly in cuboidal epithelial cells of collecting tubules.

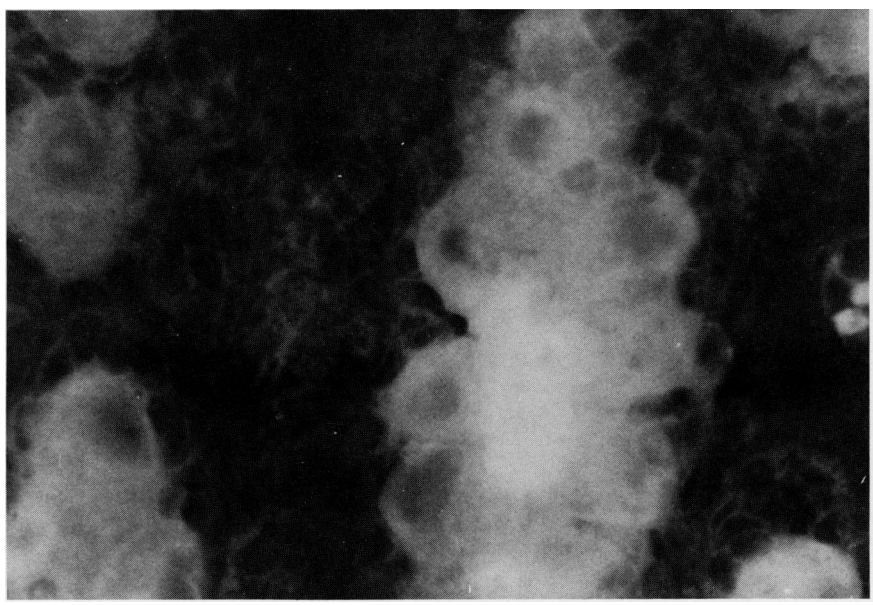

FIG. 1. Detection of human CMV in the kidney by anti-complement immunofluorescence (ACIF). The frozen section of kidney was fixed with cold acetone for 10 min and rinsed with phosphate buffer three times. Diluted heat-inactivated anti-CMV serum (strain AD-169) together with fresh human complement was applied. The reaction was carried out at 37° C in a moist CO_2 incubator for 45 min. The slide was then washed extensively. Goat antiserum to human C'3 (FITC conjugated to β_1C/β_1A globulin from Hyland, Calif.) was then applied for detection of specific complement fixation. The location of the CMV antigen was mainly in cuboidal epithelial cells of the collecting tubules (magnification about 1000 x).

The degree of specificity and the level of background fluorescence depend strongly on the sources of complement and conjugated anti-C'3 serum. Unsatisfactory commercial products are always encountered. Fractionation and removal of nonspecific contaminants by column chromatography seem to be essential for elimination of background and nonspecific reactions (27).

As shown in Fig. 7, there is heterogeneity of CMV complement fixing antigen (or antigens) among various CMV strains. Some strains have no crossing-over of CF antigen. In view of this important finding we have pooled eight strains of hyperimmune sera, prepared in the guinea pig against purified viruses, as multispecific sera which should cover a broad spectrum of human CMV for clinical diagnostic purposes.

(B) Detection of Human CMV Genome by Nucleic Acid Hybridization Techniques

As with herpes simplex virus, human CMV has a genome size around 10^8 daltons. A genome this size is sufficient to code for 200–300 virus-specific proteins of which only 15–20% may be structural proteins associated with purified virions (20). Hyperimmune sera against purified virions may not be able to detect the virus in unexpressed or latent states similar to the situation found with Raji cells that carry EBV DNA (32, 36). As detected by DNA–DNA reassociation kinetics analysis, we have found that all human CMV strains tested to date share at least 80% homology in DNA sequence (Fig. 5). We performed nucleic acid hybridization in parallel with tissue culture isolation and immunologic methods for detection of CMV.

Three kinds of nucleic acid hybridization techniques are available for human cytomegaloviruses: (i) complementary RNA (cRNA)–DNA hybridization on nitrocellulose membranes; (ii) *in situ* cRNA–DNA cytohybridization; and (iii) DNA–DNA reassociation kinetics analysis. These techniques require highly specific radioactively labeled viral cRNA or *in vitro* labeled viral DNA as probes. In order to prepare the viral DNA to serve as template for the synthesis of virus-specific cRNA or *in vitro* labeled viral DNA, we use only purified extracellular virus. The virus (strain AD-169) was concentrated from the clarified supernatant fluids of infected cells and purified through sucrose and preformed CsCl gradient centrifugation as described previously (20). The viral DNA was released from the purified virus by treating with sodium deodecyl sulfate (SDS) and pronase. The DNA was then purified by sedimentation through 10–30% sucrose gradients and by two cycles of isopycnic centrifugation in cesium chloride (20).

(C) Synthesis of Human CMV-Specific ^3H (or ^{125}I) cRNA

E. coli DNA-dependent RNA polymerase (transcriptase) is used for synthesis of viral specific cRNA with purified viral DNA as template. This transcriptase from *E. coli* (strain Q13, RNase$^-$) is prepared according to the method of Burgess (5); the activity of the enzyme is around 200 units/ml and 400 units/mg of protein.

The CMV template DNA (2 μg in 0.12 ml), *E. coli* transcriptase, 6 units in 30 μl, and 0.1 ml of reaction mixture containing 0.1 *M* Tris–HCl, pH 7.9, 0.025 *M* $MgCl_2$, 0.25 m*M* EDTA, 0.25 m*M* dithiotheitol, 0.375 *M* KCl, 1.25 mg of bovine serum albumin/ml, 0.375 m*M* of ATP, GTP, and CTP, and 0.5 mCi of [^{3}H]UTP (30 Ci/m*M*) were mixed and kept at 37°C for 2 hr. After reaction the DNA was digested with pancreatic DNase (RNase-free, Worthington) at a concentration of 40 μg/ml for 30 min; and SDS was added to stop the reaction. The mixture was chromatographed through a Sephadex G-50 column in 0.1 × SSC and 0.01% SDS buffer. The first peak of radioactivity and absorbancy (A_{260}) was collected, extracted with phenol or chloroform three times, and dialyzed against three changes of 0.1 × SSC with 0.01% SDS at 4°C for 24 hr. The efficiency of incorporation of [^{3}H]UTP into acid-precipitable RNA was about 6% of

the total input. Complementary RNA with a specific activity between 8×10^6 and 1×10^7 cpm/μg was obtained (20).

For synthesis of [^{125}I] cRNA, 5-[^{125}I]Iodo-CTP and cold UTP were used to replace CTP and [^{3}H]UTP in the above reaction mixture. The details of the synthesis of 5-[^{125}I]Iodo-CTP have been described previously (38).

(D) Complementary RNA-DNA Hybridization on Nitrocellulose Membrane Filters

Total DNA was extracted by pronase–SDS from cells or tissues thought to harbor viral nucleic acid (20). The DNA was denatured with alkali and immobilized on nitrocellulose membrane filters (Schleicher and Schuell), and radioisotopically labeled CMV cRNA was applied. The hybridization was carried out by the method of Gillespie and Spiegelman (12) and as described before (20) at 66° C in 6 × SSC for 16 to 20 hr. The filters were then extensively washed and treated with pancreatic RNase to remove nonhybridized cRNA. Radioactivity above the nonspecific background level indicates that a specific hybridization of the radioactive cRNA to complementary DNA sequences in the extracted DNA has occurred, and viral DNA must be present in the tissue. Not only can viral DNA, whether encapsidated or nonencapsidated, be detected, but also a quantitative estimation of the number of viral genomes can be made.

Figure 2 shows the reconstruction curve which provides the basis for the viral genome calculations. This curve was constructed based on the amount of radioactive CMV cRNA hybridized to a graded amount of CMV DNA in the presence of 50 μg of HEp-2 or calf-thymus DNA. If the molecular weight of CMV DNA and diploid human cell DNA are 10^8 and 4×10^{12} (20), respectively, then 0.1 μg of CMV DNA in 50 μg of human tissue DNA is equivalent to 80 CMV genomes per cell. The counts of cRNA hybridized to 0.1 μg of CMV DNA in the presence of 50 μg of HEp-2 DNA is 3882 cpm which is equal to 48 cpm/genome/cell. By this calibration we should readily detect as few as two genome equivalents per cell. Detection of fewer genome equivalents would be unreliable because of the usual background of nonspecific

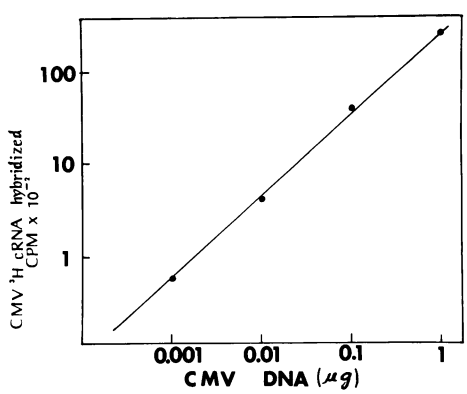

FIG. 2. Membrane hybridization of CMV [^{3}H]cRNA to CMV DNA. Graded amounts of CMV DNA (0.001–1 μg) were mixed with 50 μg of HEp-2 DNA (in 0.1 × SSC) and denatured in 0.5 *N* NaOH for 2–3 hr at 37° C. The DNA solution was neutralized with 1.1 *N* HCl in 0.2 *M* Tris, adjusted to 6 × SSC, and immobilized on nitrocellulose membrane filters. The input cRNA for each filter was 1.5×10^5 cpm (sp act is about 10^7 cpm/μg). The amount of DNA retained on the filter, determined by a diphenylamine test after hybridization, remained constant. The background hybridized counts for 50 μg of HEp-2 DNA (130 cpm) were subtracted from each value.

hybridization. In this case the more sensitive DNA–DNA reassociation kinetics analysis should be applied if the quantity of sample DNA is sufficient.

(E) Complementary RNA–DNA Cytohybridization in situ

A potentially useful technique for viral diagnostic work is known as cRNA–DNA cytohybridization *in situ*. This test is derived and modified from classic cRNA–DNA membrane hybridization. However, in this method the cellular DNA is not extracted; rather, the hybridization of radioactive labeled cRNA to DNA is carried out directly in fixed cells or specimens. The site at which the specific hybridization occurs can be localized and detected by autoradiography.

This method is adapted from that of Gall and Pardue (11) and has been successfully used for the human cytomegalovirus system (20, 34).

Frozen sections (6 to 10 μ in thickness) or exfoliated cells, virus-infected tissue-culture cells, or other specimens were applied to the slide and fixed with freshly prepared ice-chilled fixative (ethanol, 3 parts, and acetic acid, 1 part) for 10 to 15 min. After dipping in 95% alcohol (ETOH) and absolute alcohol (ETOH) to remove the residual acetic acid, the slides were then dipped into 0.4% agarose at 60° C and air dried to form a thin agarose layer on the slide to prevent detachment of cells.

The denaturation of DNA was carried out by alkalinizing the specimens in 0.07 *N* to 0.1 *N* NaOH for 3 min. The slides were then washed extensively with 70% alcohol (ETOH) and absolute alcohol (ETOH) and air dried.

One-tenth of a milliliter of CMV ^{3}H (or ^{125}I) cRNA (3×10^{5} cpm/0.1 ml with 1 mg yeast RNA, 0.1% SDS, and 6 × SSC) was applied to each slide and covered with a coverslip to prevent evaporation. The specific activity of the [3H]RNA was about 1×10^{7} cpm/μg and of the [^{125}I]RNA about 4×10^{7} cpm/μg (38). The hybridization was carried out in a moist chamber at 70° C for 20 hr. The slides were rinsed with 2 × SSC four times and treated with 40 μg/ml pancreatic RNase for 30 min at 37° C. After extensive washing again with 2 × SSC, the slides were sequentially dehydrated with 70%, 95%, and absolute alcohol and dried.

Kodak Nuclear Track NTB 2 was used for autoradiography. The exposure time for tritiated labeled material ranges from 4 weeks to 2 months, but for ^{125}I material the time is overnight to 1 week; the time depends mainly on the specific radioactivity of viral cRNA and also on the viral DNA content of the cells. Recently we have been able to shorten the exposure time of ^{3}H-labeled material from 4 weeks to 1 or 2 days by dipping the emulsion-covered slide (after complete drying) in liquid scintillation fluid–dioxane and PPO (1% w/v), and POPOP (0.02%). The detailed procedure for development and Giemsa staining was described previously (34).

Figure 3 shows an example of cRNA–DNA cytohybridization *in situ* with CMV [^{3}H]cRNA applied to kidney tissue (in collaboration with Dr. G. Nankervis). The tissue was from autopsied kidney of a congenitally infected infant with CMV infection (as described above in ACIF test). This photograph does not represent the incorporation of [^{3}H] thymidine in the DNA, but rather the hybridization of CMV [^{3}H]cRNA to the viral DNA in the cells. The gains which deposit heavily in cuboidal epithelial cells in the collection tubules represent the localization of viral DNA. As shown in Fig. 1, CMV viral structural antigen was also detected in these cells by the ACIF test.

We have used this technique to detect CMV in leukocytes and in human semen as well as in various autopsied specimens. This technique is quite reliable; the viral genome can be detected whether or not infectious virus is present. There is no need to use tissue culture. As mentioned above, one of the great advantages of this technique is that it permits not only the detection of viral genome but also its location as to the cell type in a mixed cell population and its intracellular location. However one does need virus-specific radioactive cRNA for use as the specific probe. This specific probe is not yet available commercially, and it is relatively expensive to prepare as compared to those agents used in the immunofluorescence tests.

RNA–DNA cytohybridization is not as sensitive as cRNA–DNA membrane

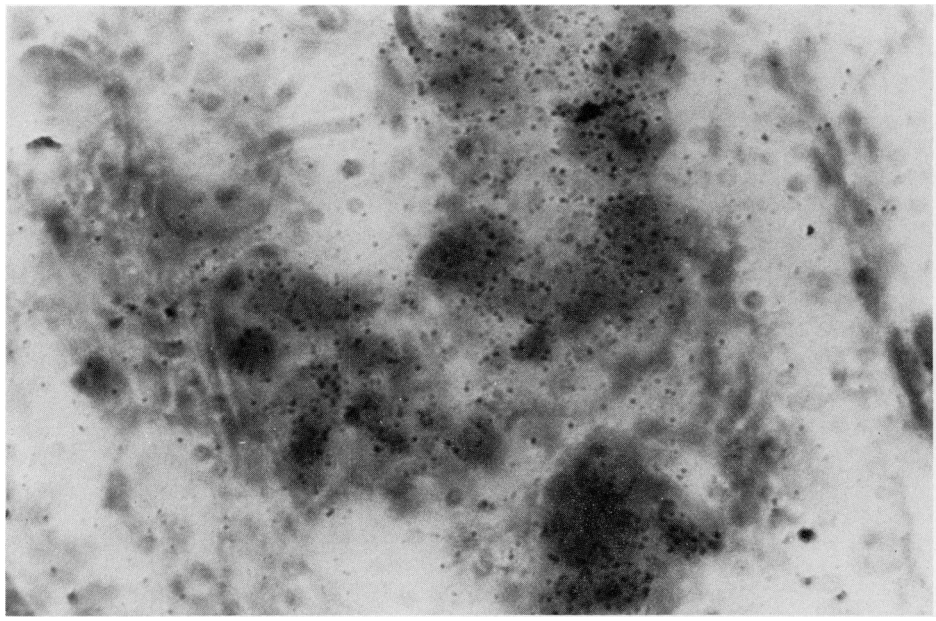

FIG. 3. Complementary RNA–DNA cytohybridization *in situ* with CMV [^{3}H]cRNA applied to kidney. A tissue block from the kidney of a congenitally CMV-infected infant was embedded in Ames O.C.T. compound. The tissue was sliced into sections 6 μm thick, transferred to slides, and fixed with freshly prepared fixative. After dehydration the section was exposed to 0.07 *N* NaOH for 3 min and dehydrated with 70% and 95% ethyl alcohol. The cytohybridization was carried out as described in the text.

hybridization because in the fomer method fewer cells are examined, whereas in the latter method the total DNA is extracted from a number of cells for hybridization and testing. But if most of the viral genomic material is confined to only a few cells, then the cytohybridization is considered to be relatively sensitive.

(F) DNA–DNA Reassociation Kinetics Analysis

Precise quantitation of viral genome in the tissue can be achieved by DNA–DNA reassociation kinetics analysis (4, 20, 25, 33, 44). This technique can detect as little as 0.2 viral genome per cell when 2 mg of total cellular DNA is used. This technique also can be used for studying genetic relatedness and interstrain homology (23). This analysis is based on the principle that under fixed conditions the kinetics of reassociation of a pair of complementary DNA strands that have been denatured and allowed to reanneal are dependent on the complexity of the DNA and the collision frequency of the complementary DNA strands. Therefore the period for the reannealing of a particular DNA should be inversely proportional to its concentration (4, 44). If we are examining a cellular DNA preparation obtained from a tissue or cell specimen with cytomegalovirus infection, the addition of this DNA into the reassociation mixture, which contains a constant amount of radioactive viral DNA probe, increases the total concentration of CMV DNA in the system. Therefore, the reannealing of the radioactive DNA probe is accelerated by the admixed DNA. From the degree of acceleration of reannealing, we can quantitate precisely the amount of viral DNA in the test specimen.

Pure viral DNA with high specific radioactivity is essential for this analysis. In view of the difficulty of obtaining viral DNA with high specific radioactivity by *in vivo*

labeling, we applied nick-translation with Kornberg's enzyme to label CMV DNA *in vitro*. The purified CMV DNA was first randomly nicked with a low concentration of pancreatic DNase I and then subjected to repair synthesis with DNA polymerase I. Through combined 3′ polymerization and 5′ exonuclease digestion, the cold TMP residues in the CMV DNA are gradually replaced by radioactive TMP. This method essentially follows that used to label Epstein–Barr virus DNA (33) and has been described in detail previously (20, 23). The specific radioactivity of *in vitro* labeled DNA obtained ranged from 1.6 to 3 × 10^6 cpm/μg.

The *in vitro* tritium-labeled CMV DNA and unlabeled test-cell DNA were fragmented by ultrasonic vibrations to about 5S as determined by preparative ultracentrifugation. The procedure for DNA–DNA reassociation followed the method of Kohne and Britten (25) modified as described previously (20, 23). In brief, 0.01 μg of probe [^{3}H]CMV DNA (about 1.6 ~ 3 × 10^4 cpm) and unlabeled test-sample DNA in 0.01 *M* Tris–HCl, pH 7.4, and 0.0025 *M* EDTA were denatured at 100° C for 15 min. After rapidly chilling in ice, the salt concentration was adjusted to 0.9 *M* NaCl, and the DNA was then allowed to reanneal at 66° C. The proportion of reassociated DNA was determined either by hydroxyapatite chromatography (25) or S_1 enzyme differential digestion (23).

As part of a study of the genetic relatedness among viruses in the herpes group we have shown the lack of detectable nucleic acid homology between a strain of human CMV (AD-169) and herpes simplex type 1 and type 2, EBV, murine CMV, and simian CMV by DNA–DNA reassociation kinetics analysis (23). Our next effort was to demonstrate by the same technique the degree of relatedness or difference between strains of human CMV. Figure 4 shows the results of an experiment in which *in vitro* labeled [^{3}H]CMV DNA (strain AD-169) was used in comparison with some unlabeled viral DNAs purified from different strains of human cytomegalovirus (Davis, Esp, and TW-087) and from a virus (Colburn) isolated from human brain; the last virus was isolated from a child with encephalitis; in tissue culture the virus resembled CMV. The results show that the DNAs from strains Davis, TW-087, and Esp do accelerate the reassociation of [^{3}H]AD-169 DNA probe with kinetics that indicate more than 80% nuclei acid homology among these human CMV strains. Colburn virus and its DNA do no resemble human CMV; the virus was found to be related to monkey CMV (manuscript in preparation).

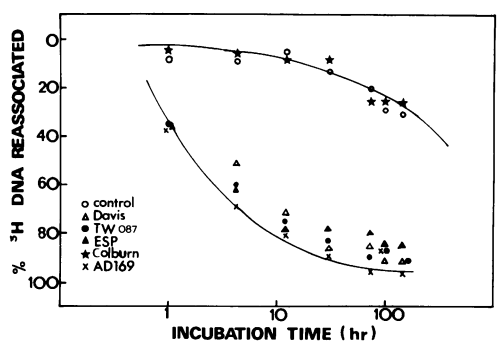

FIG. 4. Reassociation kinetics analysis of tritiated AD-169 CMV DNA with the DNA of Colburn virus and various human CMV isolates.

Sonically disrupted ^{3}H-labeled AD-169 DNA, 0.01 μg (2.7 × 10^4 cpm), 20 μg of calf-thymus DNA, and 2 μg of unlabeled viral DNA were mixed and heat denatured in the presence of 0.01 *M* Tris–HCl (pH 7.4) and 0.0025 *M* EDTA. The salt concentration was then adjusted to 0.9 *M* NaCl. The hybridization was carried out at 66° C, and the fraction of reassociated [^{3}H]DNA was analyzed by S_1 enzyme differential digestion as described above. Calf-thymus DNA control (0); Colburn DNA (★); Davis DNA (△); TW-087 DNA (●); Esp DNA (▲); and homologous AD-169 DNX (X).

III. VIRUS STRAIN VARIATION

Early in 1960 Weller *et al.* reported that human CMV was not an antigenically homogeneous group (41). Since then, more evidence indicates that human CMV has considerable antigenic diversity (42, 43). With the use of sera from congenitally CMV-infected infants, Weller *et al.* (41) examined three culture-adapted strains from congenitally infected infants and Rowe's AD–169 virus isolated from adenoid tissue by kinetics of neutralization and concluded that strains Davis and AD–169 were sufficiently distinct to be classified as type 1 and type 2. Strains Esp and Kerr were tentatively placed in a third antigenic group that exhibited some cross-reactivity with the type 1 Davis strain. In 1969 Krech and Jung (26) prepared antisera to human CMV in the guinea pig and reported that sera neutralizing the Davis strain lack activity against the ESP strain.

It has been 15 years since Weller's preliminary serological classification with patients' sera: No further attempt has been made to assign the genetic or antigenic relatedness among strains isolated from different organs or from different diseases. From the therapeutic or preventive point of view, it is urgent to have a comprehensive classification of human CMV based on antigenicity and nucleic acid homology. The selection of strains suitable for vaccination, for passive immunization, and for diagnosis will be completely based on this information. Motivated by these considerations, we have now propagated and purified 12 strains of human CMV from various sources in relatively large amounts to produce hyperimmune sera of high titer in heterologous species for antigenic study and to prepare purified viral DNAs for determination of nucleic acid homology.

Viral nucleic acid homology studies by DNA–DNA reassociation kinetics analysis and by restriction enzyme analysis combined with the immunologic data are being used to define strain variations and to approach the questions posed by the variety of pathogenic manifestations of CMV infection. Some of our preliminary observations are as follows.

(A) Cytomegalovirus Strains and Origin

AD-169, isolated from adenoid tissue by W. Rowe, Bethesda, Md., passage 285–289 (37, 41).

Davis, isolated from liver, T. Weller, Boston, Mass., passage 65–68 (41).

Kerr, from urine, U.S. Naval Hospital, Chelsea, Mass., passage 23–28 (41).

Esp, from urine, Babies Hospital, New York, passage 43–48 (41).

C-87, isolated from kidney, M. Benyesh-Melnick, Houston, Tex., passage 58–63 (2).

NC496, liver biopsy, Chapel Hill, N.C., passage 3–5.

Town, congenital infection, S. Plotkin, Philadelphia, Pa., passage 29–35 (10).

UW-1, urine, CMV inclusion disease, B. Wentworth, Seattle, Wash., passage 33–35 (6).

RCH234, Australian strain, from B. Wentworth, Seattle, Wash.

TW-087, from cervix, E.-S. Huang, Taipei, Taiwan, passage 20–24.

Clegg, from human semen, Chapel Hill, N.C.; specimen from D. Lang.

Colburn, isolated from brain tissue taken from a 6-yr-old boy suffering from an encephalopathy, obtained from C. Alford, Birmingham, Ala., passage 80–85.

(B) Interstrain Nuclei Acid Homology

DNA–DNA reassociation kinetics analysis of ^{3}H-labeled AD-169 CMV DNA with unlabeled DNA of various CMV strains (Fig. 5) shows that all the human CMV

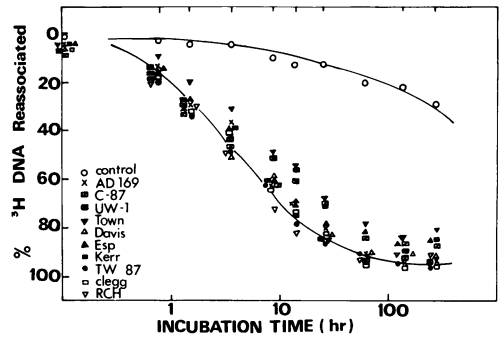

FIG. 5. Reassociation-kinetics analysis of tritiated AD-169 DNA with DNA of various human CMV strains. Sonicated [^{3}H]AD-169 DNA, 0.01 μg (2.7 $\times$ 10^4 cpm), 20 μg of calf-thymus DNA, and 2 μg of unlabeled virus DNA were mixed and denatured. The experiment was carried out as described in Fig. 4.

strains examined (Colburn is considered here not to be a human CMV strain) share at least 80% homology with AD-169 DNA. The degree of homology existing among human CMV strains is greater than that between herpes simplex type 1 and type 2, which is about 50% (24). Clegg and TW-087 are genital tract strains; both DNAs accelerated the probe [^{3}H]AD-169 DNA reassociation to the same extent as did the homologous control AD-169 DNA. These results indicate that human CMV strains do have major common nucleic acid sequences. However heterogeneity of nucleotide sequence does exist.

(C) Restriction Enzyme Analysis of Human CMV DNA

Restriction endonucleases EcoR·R-1 purified from *E. coli* strain Ry13 (15), and Hind III (40) purified from *Haemophilus influenzae* serotype *d* were used in this study. All virus DNAs were labeled with ^{32}P in infected cells, and the DNA was purified from a purified extracellular virus preparation as described previously (20). Enzyme-digested virus DNA fragments were separated in a 1% agarose slab-gel electrophoresis system. After electrophoresis the gel was vacuum-dried onto filter paper and exposed to X-ray film. Details of digestion and electrophoresis are described elsewhere (Kilpatrick, Huang, and Pagano, *J. Virology*, in press).

EcoR·R-1 or Hind III digestion of DNA from several CMV isolates yields distinct fragments for each isolate (Figs. 6A and B). Coelectrophoresis of EcoR·R-1 or Hind III digests shows considerable matching of comigrating fragments among several human isolates; however, no two isolates have identical fragment-migration patterns. The degree of matching among human isolates appears to be greater for products of EcoR·R-1 digestion than for products of Hind III among the same strains. This would make the Hind III digests a more sensitive comparison of isolates than the EcoR·R-1 digests. However, for purposes of distinguishing between human and nonhuman CMV isolates, it is expected that both enzyme products will be equally useful. Comparison of EcoR·R-1 or Hind III digests of human CMV isolates with digests of a simian isolate (GR-2757) shows little if any matching of fragments. The Colburn isolate obtained from human brain shows significant matching or comigration of fragments with simian virus GR-2757. This finding appears to be supported by DNA–DNA reassociation kinetics analysis as shown above. It is not known to what degree these digests reflect the laboratory passage history of these isolates.

Herpes simplex type 1 viruses have EcoR·R1 and Hind III digestion products distinct from the type 2 viruses; there are rare comigrating fragments (18, 39). HSV

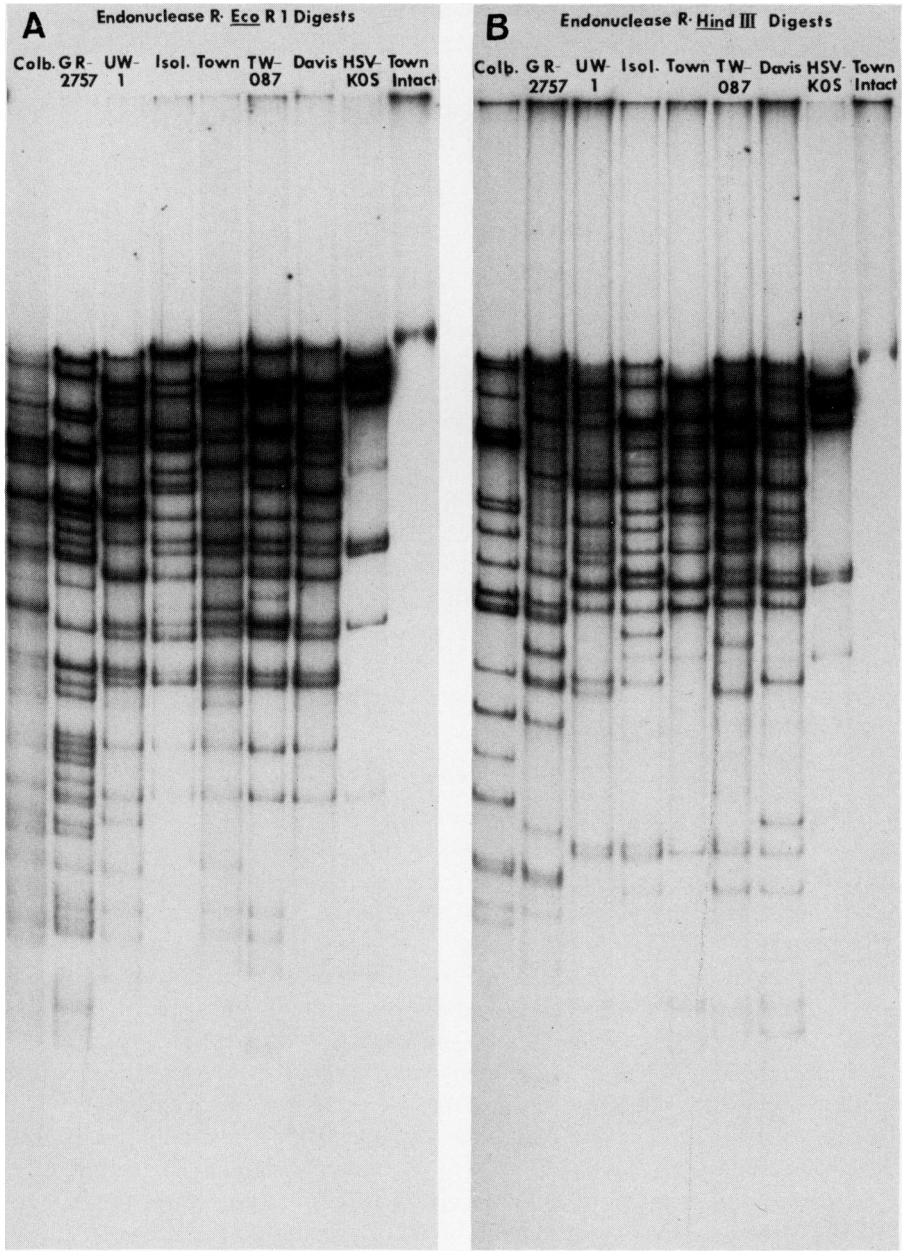

FIG. 6. Comparison and analysis of various cytomegalovirus strains by cleavage of their DNA with restriction endonucleases EcoR·R-1 and Hind III. CMV DNA labeled with ^{32}P and purified as described in the text was dissolved in TBS (0.05 *M* Tris–HCl, pH 7.4, and 0.15 *M* NaCl) and digested with either enzyme in the presence of 10 m*M* $MgCl_2$ and 5 m*M* β-mercaptoethanol for 24 hr at 37°C. All samples were electrophoresed on a 1% agarose slab gel in 1 E buffer (21) until the tracking dye (bromphenol blue) migrated to the bottom of the gel. The gels were dried and exposed to X-ray film as described (21). KOS, a strain of herpes simplex virus, was used as a marker and a control for digestion condition. Panel (A) EcoR·R-1 digests; (B) Hind III digests. KOS in both panels (A and B) was digested and generated with EcoR·R-1 enzyme.

types 1 and 2 also have only about 48% nucleic acid homology (24). However, the strains of CMV, as discussed above, show at least 80% nucleic acid homology. EcoR·R-1 or Hind III digestion of the DNAs reflect his homology, and although there are important differences, no obvious distinction into groups or classes of isolates has been found. A more detailed discussion of these digestion products will be found elsewhere (Kilpatrick, Huang, and Pagano, *J. Virology,* in press).

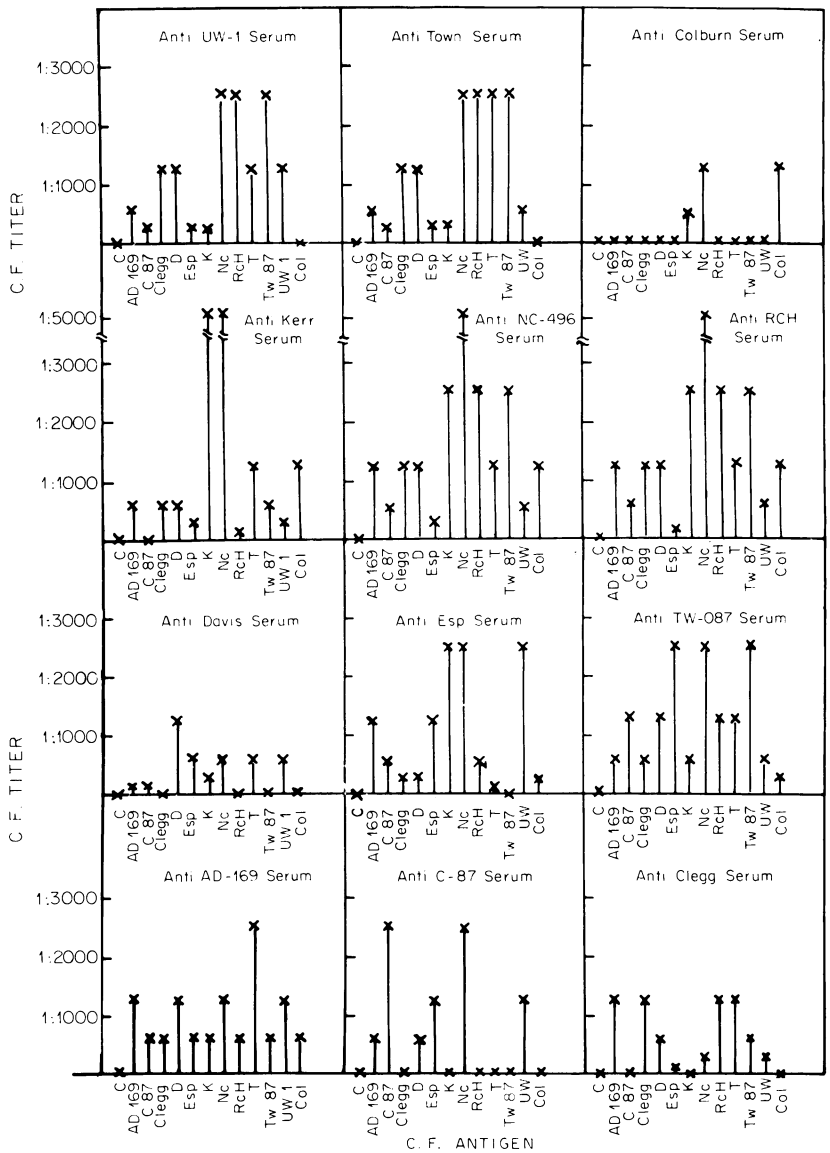

FIG. 7. Complement-fixation antibody titer of sera from guinea pig hyperimmunized to human CMV: titration against homologous and heterologous antigens. Antibody dilutions in microtiter plates containing 2 units of antigen and 2 units of complement were incubated overnight at 4° C. The method employed and preparation of CF antigen was described previously (22). Antigen "C" represents the host-cell antigen (WI-38 human fibroblast) control.

(D) Antigenic Heterogeneity among Human CMV Strains

Antisera were prepared in guinea pigs with strains of purified human CMV as published (22). The heterogeneity of the CMV strains was determined by complement fixation; immunofluorescent, indirect hemagglutination, and neutralization tests are in progress. From the CF data as shown in Fig. 7, it is clear that great heterogeneity of CF antigens do exist among those human CMV strains tested. There is no cross-reaction between C-87 and Clegg or between C-87 and Kerr, whereas there is one-way cross-reaction between several of the strains, e.g., C-87 and RCH, C-87 and TW-087, Davis and Clegg, Davis and Rowe, Davis and TW-087, Esp and TW-087, Clegg and Kerr.

Anti-NC-496 serum has a CF titer pattern similar to that of anti-RCH serum. Besides this, no identical pair can be found. The data from Fig. 7 suggest that it is difficult to classify human CMV strains into type or group according to complement-fixation pattern. Due to lack of immunologic cross-over between certain CMV strains, it is important to use multivalent hyperimmune sera when the CF test is employed for viral diagnostic purposes.

IV. DISCUSSION AND CONCLUSION

We approach the detection of human cytomegalovirus infection and the discrimination of strain differences by both nucleic acid hybridization techniques and immunological analyses. The reasons for applying nucleic acid analyses as a tool in detection and classification are first that human CMV antigen has such a large diversity that detection or analysis of the structural antigens alone cannot delineate all viral genetic information, and second that some viral information may not be expressed as a structural antigen.

As shown in the DNA–DNA reassociation kinetics analysis, human cytomegalovirus isolates share at least 80% of nucleic acid sequences in common. The difference in nucleotide sequence among these human CMV strains is obviously less than the difference between herpes simplex virus type 1 and type 2 (18, 24). Restriction endonuclease cleavage patterns of these human CMV strains show the heterogeneity of the genome structure, but these strains do share common comigrating fragments. These comigrating fragments observed after EcoR·R-1 or Hind III cleavage and gel electrophoresis might be constant regions responsible for common CMV biological characteristics and possibly could serve as markers for virus identification purposes.

Preliminary study of the genital strains isolated from semen and cervix, Clegg and TW-087, do not disclose any great distinction from other human CMV isolates by reassociation kinetics analysis and restriction endonuclease analysis (manuscript in preparation) as exists between herpes simplex virus type 1 and type 2 (18, 24, 35). Before final conclusions can be made, further investigation is necessary.

In analyses of restriction enzyme cleavage and electrophoresis patterns of Town strain (10) DNA, we do not find any significant variation between passage 30 and 36. With a given restriction enzyme the resultant cleavage pattern is very consistent with low multiplicity infections (MOI is about 1 PFU). Any major change in the cleavage sites might occur when the viruses are first subjected to the tissue culture cultivation system. To resolve this question, we intend to examine virus collected directly from urine having a high virus titer.

Although we do not yet have complete information from neutralization kinetics analyses and immunofluorescence tests, the data obtained from complement fixation

tests with hyperimmune sera prepared from purified virus show that great heterogeneity of CF antigen exists among various human CMV isolates. There is no crossing-over between several strains.

Combining the data obtained from nucleic acid hybridization, restriction enzyme analysis, and complement fixation, we still find it quite difficult to classify human CMV isolates into types or subgroups; each isolate can serve as a type. As Weller stated, this is an ubiquitous agent, pantropic in character with great antigenic and genetic diversity.

As shown above, the nucleic acid hybridization techniques are very useful for detection of human cytomegalovirus. Due to their expense and the complicated techniques, these procedures may be only suitable for research purposes at the present moment. They are of great value in providing fundamental information on the pathobiology of CMV infection.

V. SUMMARY

Detection and localization of human cytomegalovirus antigens and viral genomes was accomplished by nucleic acid hybridization techniques (cRNA–DNA membrane hybridization, cRNA–DNA cytohybridization *in situ*, and DNA–DNA reassociation kinetics analysis) and anti-complement immunofluorescence tests. Study of interstrain genetic relatedness by DNA–DNA reassociation kinetics analysis shows that the human CMV isolates examined share at least 80% homology with AD-169 DNA. Restriction endonuclease cleavage of DNA from several CMV isolates shows considerable comigration of fragments. However, no two isolates have identical fragment-migration patterns. The heterogeneity of the antigens of the CMV isolates was also demonstrated by complement fixation with antisera prepared in guinea pigs against purified preparations of human CMV isolates. Thus far it is difficult to classify human CMV strains into types or groups, although important differences seem to exist.

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