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Comparison of the Polypeptides of Several Strains of Human Cytomegalovirus

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SUMMARY

Analysis of purified human cytomegalovirus (CMV) by sodium dodecyl sulphate-polyacrylamide gel electrophoresis revealed 32 polypeptides with mol. wt. ranging from 13500 to 235000. Similar analysis of purified preparations of four strains of CMV showed a remarkable similarity in polypeptide composition. Results indicate that the four strains may be related.

INTRODUCTION

Human cytomegalovirus (CMV) is morphologically similar to herpes simplex virus (HSV: Smith & Rasmussen, 1963; Wright, Goodheart & Lielausis, 1964), but differs biologically from HSV. For example: (1) CMV and HSV are not antigenically related (Martos *et al.* 1970); (2) unlike HSV, *in vitro* replication of CMV in human embryo cells is characterized by a long eclipse period (St. Jeor & Rapp, 1973); (3) growth of human CMV is restricted to human cells but HSV can grow in human and in a variety of primate and non-primate cells; and (4) DNA-DNA hybridization analysis does not reveal homology between HSV and human CMV (Huang & Pagano, 1974), even though the mol. wt. of purified DNA from these two viruses are similar (Huang, Chen & Pagano, 1973).

There are discrepancies concerning the antigenic relatedness of various isolates of human cytomegalovirus. Results suggesting that they are antigenically unrelated have been derived using cross-neutralization tests (Weller, Hanshaw & Scott, 1960; Birnbaum *et al.* 1969). However, complement-fixation tests (Stern & Elek, 1965) and cross-neutralization tests in the presence of added complement (Haines, Von Essen & Benyesh-Melnick, 1971) suggest that various human cytomegaloviruses are serologically related. CMV DNA analysis by DNA-DNA renaturation kinetics (Huang *et al.* 1977) and restriction endonuclease suggests a genetic homology between human CMV strains (Kilpatrick, Huang & Pagano, 1976). Relatedness among different isolates of CMV can also be determined by comparing the polypeptides of the various CMV strains. Very little is known about the nature and synthesis of CMV polypeptides under *in vitro* and *in vivo* conditions. Sarov & Abady (1975) recently characterized several polypeptides of a partially purified preparation of human CMV by polyacrylamide gel electrophoresis.

In this study, we describe the characterization of human CMV polypeptides. These data include the identification of the structural polypeptides of enveloped CMV virions and a comparison of the polypeptide composition of intact virions from four CMV strains.

METHODS

Cells and viruses. Human embryo lung (HEL) fibroblast cells were grown in Dulbecco modified Eagle's medium supplemented with 10% foetal calf serum (FCS). Various strains of human cytomegalovirus were obtained from the following persons: strain AD169 from Dr M. Benyesh-Melnick (Baylor College of Medicine, Houston, Texas); strain C87 from Dr P. Feorino (Center for Disease Control, Atlanta, Georgia); strain Birch from Dr W. Rowe (NIH, Bethesda, Maryland); and strain Towne from Dr S. Plotkin (Children's Hospital, Philadelphia, Pennsylvania). The passage histories of strains AD169, C87, and Towne have been described elsewhere (Weller *et al.* 1957; Benyesh-Melnick, Rosenberg & Watson, 1964; Plotkin *et al.* 1975). The Birch strain was isolated from a patient with Hodgkin's disease (W. Rowe, personal communication). The procedures for the assay of CMV as measured by plaque forming units (p.f.u.) have been described previously (St. Jeor & Rapp, 1973).

Radioactive labelling of cytomegalovirus. Confluent monolayers of HEL cells grown in roller bottles were infected with the virus at an input multiplicity of 0·2 to 0·5 p.f.u./cell. Following virus adsorption for 2 h at 37 °C, Dulbecco modified Eagle's medium supplemented with 5% FCS was added to the cultures. When 10% of the cells showed cytopathology, usually at two days post-infection (p.i.), medium was replaced with labelling medium containing 5 μCi/ml ^{35}S-methionine (sp. act. 360 Ci/mmol, Amersham/Searle, Inc., Arlington Heights, Illinois). Labelling medium was a reconstituted Eagle's medium deficient in unlabelled methionine containing 5% dialysed FCS. The virus was isolated from the medium when complete cytopathology was observed, usually on the fifth or sixth day p.i. At this time, virus infectivity in the infected cell medium reached a plateau and the titres obtained were similar to that obtained with normal growth medium, that is, from 1×10^6 to 8×10^6 p.f.u./ml.

In preparing an uninfected control, HEL cells were labelled with ^{35}S-methionine for a similar period by the described procedure. To establish purification criteria, HEL cells were prelabelled with ^{35}S-methionine (final concentration 5 μCi/ml) for 24 h in the labelling medium described above. Following incorporation, the labelling medium was removed and the cell sheet was washed three times with unlabelled Dulbecco modified Eagle's medium and was further incubated for 24 h. The medium was then removed and the cells were infected with the virus as described above. Following a 2 h adsorption period, unlabelled Dulbecco modified Eagle's medium was added to the cells. Virus was harvested at 7 days p.i. when all cells exhibited typical CMV cytopathology.

Purification of virus. Medium was removed from the cultures and clarified by centrifuging at 750 ***g*** for 20 min at 4 °C. Following initial clarification, virus was pelleted from the supernatant fluid by centrifuging at 27000 ***g*** for 3 h. The virus pellet was then dispersed in 0·02 M-tris, 0·15 M-NaCl, and 0·01 M-$MgCl_2$ (pH 7·5) by sonication (Branson Automatic Cleaner, Cole Parmer Instrument, Niles, Illinois) for 2 min. The resulting virus suspension was treated with 100 μg/ml purified DNase-I (Sigma Chemical Company, St Louis, Missouri) at 37 °C for 30 min and centrifuged at 8000 ***g*** for 10 min to remove cellular debris. The supernatant fluid was applied on to discontinuous sucrose gradients and prepared by successive layering of 60% (w/v), 45% (w/v), and 22% (w/v) sucrose in phosphate-buffered saline (PBS). These gradients were centrifuged at 38000 rev/min for 2 h at 4 °C in an SW41 rotor. Bands collected from the interface of 22 to 45% and 45 to 60% by a syringe from the top were dialysed against PBS and examined by electron microscopy. Trichloroacetic acid (TCA)-precipitable radioactivity was also measured for each band. Enveloped CMV typic-

Table 1. *Purification of* ^{35}*S-methionine-labelled human cytomegalovirus (Towne)*

	Radioactivity/50 μl*		Prelabelled cells	Infected / Uninfected
	Infected	Uninfected		
Medium†	54320	59450	—	0·91
20–45 % interface‡	30000	300	150	100
45–60 % interface	6000	200	100	30

* 50 μl of each sample was spotted on Whatman filter paper and TCA-precipitable radioactivity was determined.

† Medium is the supernatant from the extracellular fluid after centrifugation at 750 *g* for 20 min.

‡ Material banded at the interface of 20 to 45 % and 45 to 60 % sucrose gradients was collected and dialysed to remove sucrose.

ally bands at the interface of 22 to 45 % sucrose. Further attempts to purify this band by CsCl gradient centrifugation (Huang *et al.* 1973) or by potassium tartrate gradient sedimentation (Spear & Roizman, 1972) did not improve the purity of the virus preparation as determined by electron microscope examination. In purifying large batches of virus, a second sedimentation in CsCl (Huang *et al.* 1973) was found to be helpful in removing cellular contaminants. Use of a linear sucrose gradient of 20 to 60 % (w/v) in place of the discontinuous sucrose gradient described did not improve the purification. Extracellular medium from uninfected cells and prelabelled cells infected with CMV were also processed by the procedure described above for virus purification.

Electrophoresis and autoradiography of gels. Labelled purified virus was precipitated by adding TCA to a concentration of 10 % in the presence of 50 to 100 μg carrier protein. After incubation at 0 °C for at least 1 h, the mixture was centrifuged at 15000 *g* for 30 min. The pellet was washed twice with acetone, dissolved in a buffer containing 0·0625 M-tris, 2 % sodium dodecyl sulphate (SDS) and 5 % mercaptoethanol, and heated at 100 °C for 2 min. Analysis of the solubilized proteins was carried out by 5 to 15 % (w/v) gradient polyacrylamide slab gel electrophoresis as described by Baum, Horwitz & Maizel (1972). Following electrophoresis, the gel was dried and an autoradiogram was prepared using Kodak no-screen X-ray film.

RESULTS

Isolation of cytomegalovirions

We have described a simple procedure for the purification of cytomegalovirus. The problem of host protein contamination in purifying HSV was avoided by purifying virus derived from extracellular fluid. An estimate of the enrichment of virus proteins with respect to host proteins during the course of purification was made by labelling a parallel culture of uninfected HEL cells with ^{35}S-methionine and determining the ratio of radioactive banding at the 22 % and 45 % interface obtained from the infected and uninfected cells. Data from a typical experiment using the Towne strain of CMV are shown in Table 1. The total degree of purification indicated by this experiment was 100-fold. Electron microscope examination of the virus which banded at the interface of 22 to 45 % revealed the presence of predominantly (70 to 80 %) enveloped virus with very little apparent cellular contamination. Analysis of CMV purified from prelabelled host cells showed low levels of radioactivity (Table 1), indicating the presence of very little cellular contaminating protein in the purified CMV. The amount of virus recovered from the prelabelled host cell was similar to that recovered from infected cells grown continuously in the labelling medium. Labelling the

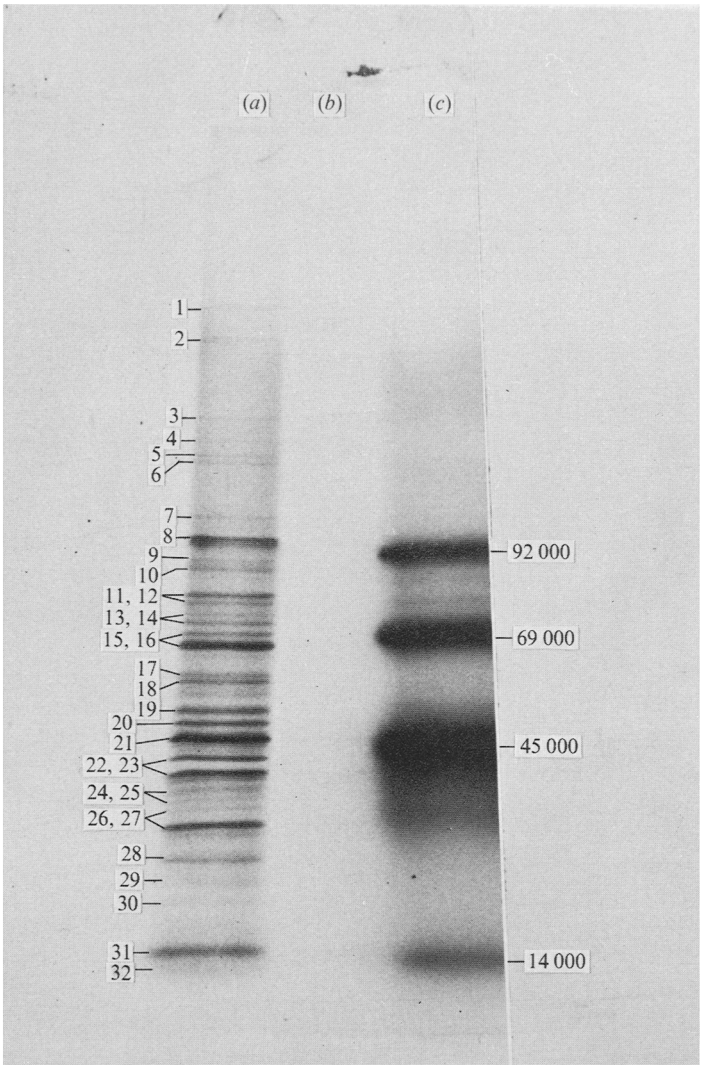

Fig. 1. Autoradiogram of ^{35}S-methionine-labelled CMV (Towne strain) polypeptides separated on a 5 to 15 % gradient polyacrylamide slab gel in the presence of SDS. (*a*) Polypeptides from purified virions. (*b*) Polypeptides from uninfected control prepared by the same procedure as described for the purification of virus. (*c*) Standard proteins of known mol. wt. are indicated.

virus with ^{3}H-thymidine demonstrated that the majority of the DNA was associated with this virus band (data not shown). This observation confirms the presence of virus in this band, since the dense body does not contain DNA (Sarov & Abady, 1975).

Polypeptide composition of human cytomegalovirus

The polypeptide composition of purified (22 to 45% interface band) cytomegalovirions (Towne strain) was analysed by SDS-polyacrylamide gel electrophoresis (Fig. 1). An autoradiogram prepared from the gel shows that CMV contains at least 32 polypeptides. To determine which polypeptides are derived from the host cell as contaminating proteins, appropriate sucrose bands (22 to 45% interface) prepared from labelled uninfected cells

Table 2. *Polypeptide composition of human cytomegalovirions*

Polypeptide (VP)	Mol. wt. ($\times 10^{-3}$)*	Origin†
1	235	VP
2	205	VP
3	150	VP
4	142	VP
5	135	VP
6	130	VP
7	105	VP
8	98	VP
9	91	VP
10	85	VP
11	78	VP
12	75	VP
13	73	VP
14	70	VP
15	68	VP
16	65	VP
17	58	VP
18	57	VP
19	50·5	VP
20	48	VP
21	45	?
22	40	VP
23	37	?
24	34	VP
25	32	VP
26	31	VP
27	28	?
28	23·5	VP
29	21	VP
30	19·5	VP
31	15·0	?
32	13·5	VP

* Mol. wt. is calculated from the electrophoretic mobilities of standard proteins of known mol. wt. electrophoresed in the adjacent lane of the same gel.

† ? indicates unknown origin (perhaps contaminating proteins).

(see Methods) were electrophoresed in an adjacent lane. There were no significant polypeptides visible in the uninfected lane, except some minor bands having electrophoretic mobilities similar to polypeptides 21, 23, 27, and 31 of purified CMV. This observation indicates that these polypeptides of purified CMV may be derived from contaminating host proteins. Because of the low amount of radioactivity, we did not use polyacrylamide gel electrophoresis to analyse CMV isolated from prelabelled host cell as it would not be detected and could not have influenced the results presented above.

The mol. wt. of the CMV polypeptides were calculated from their migration in SDS-polyacrylamide gel relative to the known mol. wt. protein marker which was electrophoresed in an adjacent lane (Table 2). The mol. wt. ranged from 13500 to 235000.

Preliminary experiments with purified ^{14}C-glucosamine-labelled virus indicate that polypeptides 1, 5, 8, 12, 15, 17, 20, and 21 are glycoproteins (unpublished results).

Comparison of virion polypeptides of several CMV strains

Autoradiograms of electrophoretically separated virion polypeptides of independently purified preparations of four CMV strains (i.e. Birch, AD169, C87, and Towne) are shown

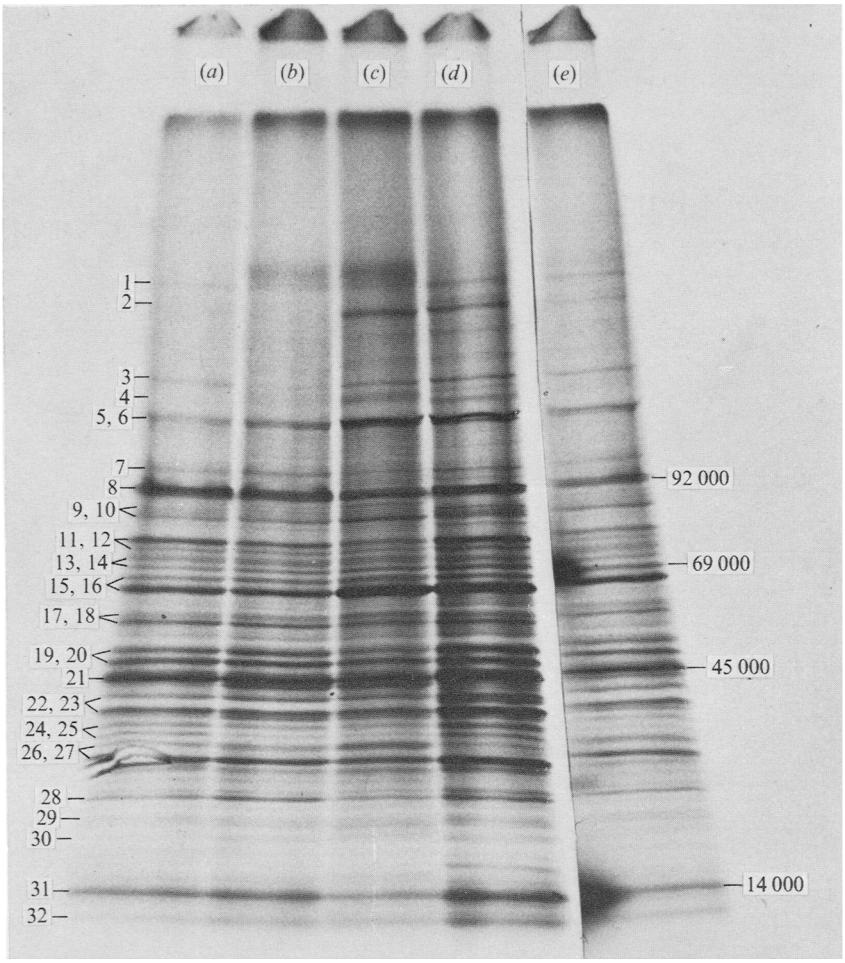

Fig. 2. Autoradiogram of a polyacrylamide slab gel containing electrophoretically separated polypeptides from purified cytomegalovirions of strain Birch (lane *a*), strain AD169 (lane *b*), strain C87 (lane *c*) and two independently purified preparations of strain Towne (lanes *d* and *e*). Proteins of known mol. wt. (14000 to 92000) were electrophoresed simultaneously in an adjacent lane. Relative mobilities are indicated. All virions are labelled with ^{35}S-methionine.

in Fig. 2 (lanes *a* to *d*). The main feature of these data is that, with few exceptions, the polypeptide compositions of four virions cannot be differentiated with respect to the relative amounts and electrophoretic mobilities of the virion polypeptides. Both VP2 and VP4 are absent in strains Birch and AD169, whereas VP1 and a faint band between VP21 and VP22 (which could not be completely resolved) are absent in strain Birch only. In addition, some very minor differences in polypeptide composition between VP8 and VP9 among the strains are noticeable. As a control, a separate batch of Towne strain purified from a different lot of HEL cells was included (lane *e*) to determine whether the polypeptide composition varied with separate preparations of the same virus strain. The similarity between the two preparations of the Towne strain indicates that the polypeptide pattern does not vary between separate preparations of the same virus. Thus, it is concluded that the polypeptide compositions of these four virus strains are very similar.

DISCUSSION

Very little is known about the replication of CMV *in vitro* and *in vivo*. To study the replication of cytomegalovirus, it is necessary to characterize the polypeptides of the virion. Sarov & Abady (1975) have reported that polypeptides contained in CMV form 19 bands when electrophoresed in SDS-polyacrylamide gel. This report has suffered serious criticism for the absence of control experiments indicating which, if any, of these polypeptides are contaminating host proteins. In this paper, we report that we have resolved several additional species of polypeptides by the use of 5 to 15% gradient polyacrylamide gel electrophoresis and have been able to identify the polypeptides which may arise from host cell contamination. The polypeptide compositions reported in Table 2 are generally comparable to those obtained by Sarov & Abady (1975), with the exception that we have detected a few very large polypeptides (mol. wt. larger than 200000) which were not detected in their polyacrylamide gel electrophoresis profile of CMV. In addition, we have resolved the virion polypeptide in much more detail than has been reported by Sarov & Abady (1975). Although we have detected a major virion polypeptide (VP16) with a mol. wt. of 65000, this polypeptide apparently does not comprise 20% of the total virus protein as has been reported by Sarov and Abady (1975). The reason for this discrepancy is unknown. However, because of the absence of a relatively homogeneous mol. wt. marker of approx. 200000, the mol. wt. of polypeptides VP1 through VP6 could not be accurately determined.

Comparison of the CMV polypeptides with those of the uninfected control suggests that some polypeptides may be contaminating host proteins (Table 2, Fig. 1), although it is also possible that polypeptides which have the same electrophoretic mobilities in CMV and in the uninfected control are different proteins of similar mol. wt. If this is the case, they may represent true virion structural proteins. Although CMV DNA (mol. wt. 100×10^6; Huang *et al.* 1973) contains the genetic information to code for this number of proteins (total estimated mol. wt. is approx. $2{\cdot}2 \times 10^6$), it is not clear whether all of these proteins are coded for by the virus genome.

Comparison of four strains of CMV showed remarkable similarity in polypeptide composition. This is in agreement with similarities observed in other biological properties, such as heat inactivation, growth cycles, and ultraviolet inactivation (C. Reed & F. Rapp, unpublished data); it is in contrast, however, with the observation made with HSV-1 in which significant variability in the polypeptide composition among different strains of HSV-1 was found (Heine *et al.* 1974). The similarity in polypeptide composition suggests that the strains may be antigenically related. Since these four strains were isolated and purified independently, one would expect to see a variation in the polypeptide pattern arising from the presence of different amounts of contaminating host protein. The identical pattern of polypeptide composition among these strains indicates that those polypeptides which have the same electrophoretic mobilities in CMV and uninfected HEL control preparations may be authentic virion polypeptides. The similarity in polypeptide composition among these four strains of CMV is also in agreement with the observation of Kilpatrick *et al.* (1976), who found 80% homology in CMV DNA among strains AD169, Towne, and C87.

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