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HCMV Envelope Antigens Induce Both Humoral and Cellular Immunity in Guinea Pigs[1] (41796)

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Abstract. Antibody and cellular immunity were measured in guinea pigs immunized with whole virion, with nucleocapsids of human cytomegalovirus or with solubilized antigens containing virus envelope proteins. All the three types of immunogens induced the production of humoral antibody as well as cytomegalovirus (CMV)-specific cellular immunity. In immunization experiments envelope antigen was as effective as immunization with whole virion.

Human cytomegalovirus (HCMV) is an ubiquitous virus, involved in many disease syndromes, for which no treatment or prophylaxis is of proven value (1). Although clinical trials of live HCMV vaccine have been carried out by us and others (2, 3), there have been few studies of immunogenic characteristics of HCMV proteins and of the viral components that protect against infection (4). In view of concerns expressed by some concerning latency and oncogenicity of HCMV (5, 6), the development of a DNA-free inactivated vaccine is desirable for comparative studies. We describe here the humoral and cellular immune responses of guinea pigs immunized with the HCMV envelope (EN), whole virion (WV), or with HCMV nucleocapsid (NC) antigens and the results of efforts to characterize their immunogenicity and specificity.

Materials and Methods. *Virus.* The Towne cytomegalovirus (CMV) strain, which has been well characterized (7, 8), was propagated on MRC-5 human embryo lung fibroblasts. As seed virus, the Towne strain was plaque-purified, propagated once on MRC-5 cells and the clarified supernatant was stored in 1-ml fractions in liquid nitrogen until used. The viral plaque technique described by Wentworth and French (9) was used for the infectivity assay. The infectivity of seed virus was 3×10^6/pfu/ml.

Cells. MRC-5 human embryo lung fibroblasts obtained from the Medical Research Council in the United Kingdom (10), were propagated in minimum essential medium (MEM) containing 7.5% fetal calf serum. The experiments were performed using MRC-5 cells between the 25th and 40th passage level.

Immunization of guinea pig. Guinea pigs were given 0.2 ml intramuscular inoculation of the antigen preparation either mixed with an equal volume of Freund's complete adjuvant or not. The preparations contained 80 μg protein/0.2 ml antigen. Three injections were given at 2-week intervals and the animals were bled 10 days after the last immunization.

Purification of the virus and preparation of antigens. MRC-5 cultures grown in roller bottles (surface area, 490 cm^2) were infected with cell-free HCMV at a multiplicity of 0.1–1 pfu/cell and incubated for 6 to 7 days at 37°C. The culture fluids were harvested and centrifuged at 2000 rpm for 20 min. CMV was concentrated using polyethyleneglycol 6000 (PEG 6000), as described by Forghani *et al.* (11). PEG 6000 was added to the supernatant fluids to a final concentration of 5% (v/w). The suspension was kept at 4°C for 4 to 18 hr and then the virus was sedimented by centrifugation in a Beckman rotor No. 19 at 8000 rpm for 30 min. The pelleted virus was suspended in 0.05 *M* Tris-buffered saline, pH 7.4, and then further purified by repeated centrifugation on a gradient of 10–50% (wt/wt) sucrose for 90 min at 22,000 rpm in an SW 27 rotor. The band, which was light and dif-

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fuse, was collected and pelleted by centrifugation for 90 min at 22,000 rpm. The pelleted virus was examined by electron microscopy and used for the preparation of component antigens.

For the preparation of subunit antigens, purified HCMV was suspended in 0.05 *M* Tris-buffered saline (pH 7.2) containing 1% NP40, 4% Triton X-100, or 1% ether, and these samples were centrifuged onto a 15% sucrose cushion for 1 hr at 100,000*g*. The top fraction contained solubilized HCMV antigens (envelope antigens), and the pelleted fraction, the NC antigens. Fractions were dialysed against PBS for 3 days at 4°C.

For preparation of [^{3}H]glucosamine-, [^{3}H]thymidine-, or [^{3}H]methionine-labeled WV and envelope antigens, the same methods were used as described above except that infected MRC-5 cultures with HCMV were refed with media containing 2% dialyzed fetal calf serum and isotope ([^{3}H]glucosamine 10 μCi/ml, [^{3}H]methionine 15 μCi/ml, and [^{3}H]thymidine 5 μCi/ml) at 24 hr postinfection. Culture fluids were harvested at the time showing more than 80% CPE (usually 5 or 6 days postinfection).

Lymphocyte proliferation responses. The proliferation response of guinea pig lymphocytes to HCMV was studied by a modification of the method described previously for human lymphocytes (12). Heparinized blood was obtained by cardiac puncture and subjected to centrifugation on Ficoll–Hypaque gradients. The mononuclear cells obtained were washed two times and resuspended at a concentration of 8×10^6 viable cells/ml in RPMI-1640, supplemented with 2% heat-inactivated CMV seronegative human serum, penicillin (125 units/ml), gentamicin (62.5 μg/ml), and 5×10^{-5} *M* 2-mercaptoethanol. The cell suspension was dispensed in 50-μl aliquots (4×10^5 cells) into round-bottom microtiter plate wells (Costar). Various dilutions of CMV virion, envelope or infected-cell sonicate antigen, and control antigen consisting of uninfected MRC-5 cell sonicate were added in 50 μl aliquots. The cultures were incubated for 6 days at 37°C in a 5% CO_2 atomosphere, 18 hr prior to harvest and 1 μCi of [^{3}H]thymidine was added to each well. The cultures were harvested with a multiple-sample harvester and processed for liquid scintillation counting.

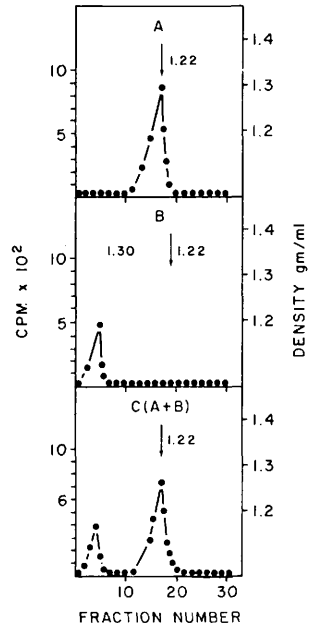

FIG. 1. Analysis of NP40-treated HCMV in CsCl gradient centrifugation. HCMV labeled with [^{3}H]thymidine was purified and treated with NP40 as described under Materials and Methods. Whole virion (A), NP40-treated virion (B), and mixture of both (C) are centrifuged in preformed CsCl gradient (density 1.34–1.17 g/ml) at 35,000 rpm for 5 hr in an SW 40.1 rotor.

Results were expressed as the stimulation index defined as counts per minute (cpm) in CMV antigen-stimulated cultures divided by cpm in control antigen-stimulated cultures.

Antibody determination. Antibody levels were quantitated in a complement-fixation assay using alkaline buffer-extracted CMV-infected cells and uninfected cells treated in the same way as antigens (13); in a neutralization test using 60% plaque reduction (14) and in an indirect immunofluorescence assay. To detect antibodies against intracellular late antigen by immunofluorescence test, infected MRC-5 cells grown on coverslips were fixed in acetone on the third days after infection, when the cytopathic effect was generalized. To detect antibodies against early intracellular antigens, infected cells were incubated in the presence of 15 μg/ml cytosine-arabinoside and fixed 24 hr after infection. A membrane immunofluorescence test was carried out on unfixed cells, on the third day after infection.

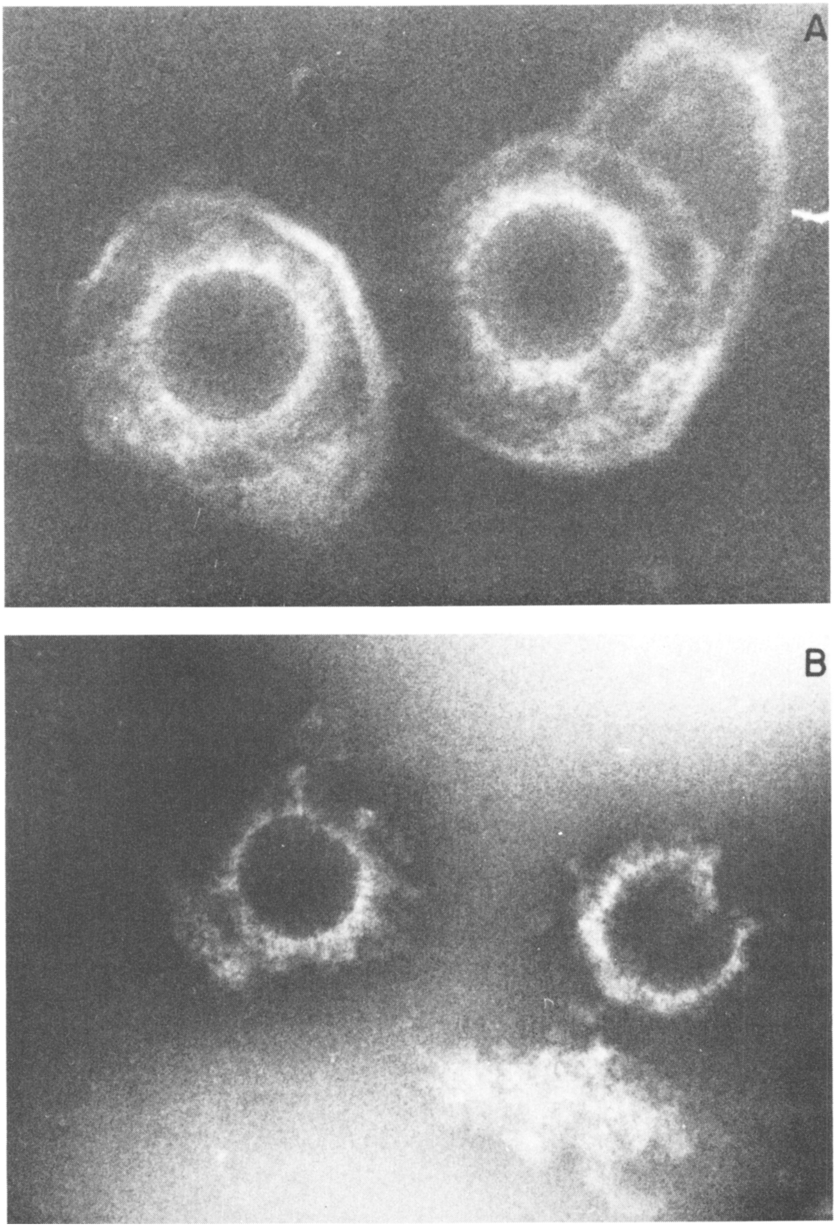

FIG. 2. (A) Negative staining of whole virion. (B) Negative staining of NP40-treated virion in which no envelope remains. ×105,000.

Guinea pig sera in various dilutions and FITC-labeled anti-guinea pig conjugate (Cappel Laboratories) in a dilution of 1:30 were used throughout.

Protein determination. Protein content was assayed by the method of Lowry *et al.* (15) using bovine serum albumin as the standard. The protein content of samples containing nonionic detergent was determined according to a modified Lowry procedure (16).

Polyacrylamide gel electrophoresis. Slab polyacrylamide gel electrophoresis was performed using a 10% sodium dodecyl sulfate (SDS) gel as described by Stinski (17) according to a modification of the Laemmli method (18). The treated samples were mixed with 4

TABLE I. BIOCHEMICAL CHARACTER OF SOLUBILIZED HCMV ANTIGEN

Sample	Protein (μg/ml)	[^{3}H]Thymidine (cpm)	[^{35}S]Methionine (cpm)	Infectivity (pfu)	Number of particles	[^{3}H]Glucosamine (cpm)
Purified whole HCMV	2100	8.0×10^4	1.3×10^5	2.5×10^8	8×10^{11}	6.5×10^5
Envelope fraction	400 (19.0)[a]	304 (0.38)	5600 (4.3)	None	None	2.5×10^5 (38)

[a] Data in parentheses are given as the percentage of cpm remaining in envelope antigen.

μl of 0.004% bromophenol blue dye. Thyroglobulin (160,000), ovalbumin (43,000), and cytochrome C (11,7000) were used as molecular weight markers. Gels were fixed for 15 min in acetic acid–5% glycerol, dried in gel drier, and autoradiographed with Kodak Tri-X Orthofilm at −70°C.

Immunoprecipitation assays. [^{35}S]Methionine-labeled HCMV was solubilized in phosphate-buffered saline (PBS) containing 0.5% NP40 and 0.5% dithiothreitol at 4°C for 1 hr. The virus lysate was then centrifuged for 30 min at 100,000*g* to remove aggregates and insoluble products. Immunoprecipitation reactions consisted of 50 μl of PBS, 50 μl of virus lysate (10^5 cpm), and 50 μl of immunized guinea pig serum. The antibody mixture was incubated for 1 hr at 4°C, followed by adding 2 mg of *Staphylococcus aureus* Cowan I protein A (SAC-I) prepared as described previously (19). The SAC-I was collected by centrifugation at 1000*g* for 20 min through a cushion of PBS containing 5% sucrose and 3% NP40, and then washed three times in PBS containing 0.5% NP40. Immune complexes were eluted from the SAC-I by incubation at 100°C for 2 min in 0.05 *M* Tris–HCl (pH 6.8) buffer, 3% SDS, 5% 2-mercaptoethanol, and 10% glycerol in 0.05 *M* Tris–HCl (pH 6.8). Electrophoretic analysis was as described above.

Results. *Analysis of immunogens.* To confirm that NP40 treatment of HCMV results in the production of naked NC, purified HCMV labeled with [^{3}H]thymidine, NP40-treated virions, and a mixture of both, were centrifuged in preformed CsCl gradients. The NP40-treated NC fraction banded at a density of 1.30 g/ml whereas WV remained at a density of 1.22 g/ml (Fig. 1). WV and the HCMV NCs were then examined by electron microscopy (Figs. 2A and B).

The concentrations of protein and DNA in the HCMV EN preparation were analyzed using virions labeled with [^{35}S]methionine, [^{3}H]thymidine, or [^{3}H]glucosamine. Approximately 20% of the protein and 38% of the glucosamine in the WV was solubilized,

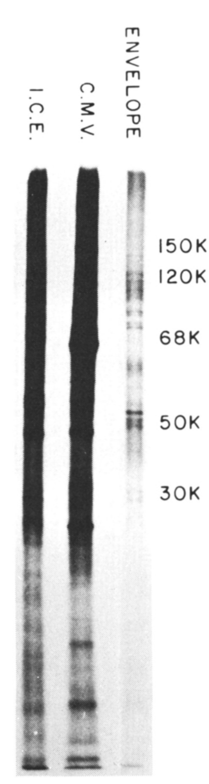

FIG. 3. Polypeptide composition of CMV-infected cells and solubilized envelope. [^{35}S]Methionine-labeled WV and EN antigens were prepared as described under Materials and Methods. Infected cells are labeled with [^{35}S]methionine (20 μCi/ml) for 2 hr at 48 hr postinfection and then homogenized to lyse the cells. Infected cell extracts were clarified by centrifugation at 18,000*g* for 15 min. These samples were prepared for SDS–polyacrylamide gel electrophoresis as described under Materials and Methods.

whereas 99% of the thymidine label remained associated with the NC fraction (Table I).

The polypeptides contained in the envelope and NC antigens prepared from [^{35}S]methionine-labeled HCMV were analyzed in 10% SDS–polyacrylamide gel electrophoresis. At least 32 bands were resolved from NC antigens, while 12 were noted in the envelope preparation (Mol-wt range 50- to 120K) (Fig. 3).

Antibody responses of guinea pigs. Preparations of WV, NC, or EN antigen were mixed with equal volumes of Freund's complete adjuvant and injected into guinea pigs as described under Materials and Methods. Antibodies were evaluated by complement fixation, indirect immunofluorescent antibody, and neutralization tests, and the results are summarized in Table II. By neutralization test, WV induced the highest antibody response, whereas NC induced the least. The envelope antigens prepared with NP40 induced a response comparable to that of WV. By complement fixation the titer varied between 8 and 128 against the CMV antigen and there was no correlation between the complement-fixing antibody titers and the immunizing antigens. However, four guinea pigs of the nine developed antibodies against the control antigen as well, in a titer of 8–64. In the immunofluorescent test the immune sera stained the cytoplasm and the membrane of the infected cells in high titer, while only the sera of the animals immunized with the WV and the NC antigen had antibodies against the intranuclear, inclusion body-like late antigens and only the sera from the NC-immunized guinea pig stained the early nuclear antigens. Considerably higher antibody level (titer between 100–800) against uninfected cells was detected in the sera using either fixed or live cells as antigens.

Development of antibodies in guinea pigs immunized with envelope antigens. To determine whether complete adjuvant is necessary to elicit antibody, and the time of antibody development, guinea pigs were immunized with or without adjuvant. Blood was taken at weekly intervals for determination of antibodies. Guinea pigs immunized without adjuvant failed to produce antibody, whereas guinea pigs immunized with adjuvant produced antibody after two successive injections.

TABLE II. ANTIBODY RESPONSES IN GUINEA PIG TO VARIOUS HCMV ANTIGENS[a]

Guinea pig	Immunizing antigen	Immunofluorescent test: Intracellular antigens: Infected cells: Early antigens: Nucleus	Cytoplasm	Late antigens: Nucleus	Cytoplasm	Noninfected cells: Nucleus	Cytoplasm	Membrane antigens: Infected cells	Noninfected cells	Complement fixation: CMV antigen	Noninfected cell antigen	Neutralization
GP-1	Virion	<10	400	50	1200	<10	400	1600	800	128	64	320
GP-A	Virion	<10	400	50	800	<10	400	800	400	N.D.	N.D.	320
GP-B	Nucleocapsid	200	200	400	1600	<10	200	1600	400	128	<8	40
GP-C[b]	Envelope	<10	200	<10	1600	<10	200	800	400	8	<8	160
GP-E[b]	Envelope	<10	400	<10	800	<10	200	800	400	8	<8	160
GP-F[b]	Envelope	<10	400	<10	1600	<10	400	1600	400	128	64	64
GP-G[b]	Envelope	<10	200	<10	1600	<10	200	1600	200	16	8	128
GP-H[b]	Envelope	<10	400	<10	1600	<10	200	800	100	16	8	256
GP-K[b]	Envelope	<10	400	<10	800	<10	200	800	200	8	<8	128

[a] Data are given as reciprocal dilutions.
[b] Guinea pigs received NP40-treated envelope antigen.

TABLE III. IMMUNOPRECIPITATION OF HCMV ANTIGENS WITH GUINEA PIG SERA

Sera	Immunogens	^{35}S-Labeled HCMV envelope[b] (cpm)	^{35}S-Labeled infected cell extract[c] (cpm)	^{35}S-Labeled control cell extract[d] (cpm)
GP-A	Virion	576 (2.04)[a]	1610 (0.90)	616 (0.75)
GP-B	Capsid	732 (2.59)	1610 (0.90)	400 (0.10)
GP-C	Envelope	1280 (4.50)	1140 (0.64)	600 (0.15)
GP-M	None	98 (0.34)	680 (0.37)	488 (0.12)
PBS	None	138 (0.48)	460 (0.25)	656 (0.16)

[a] Data in parentheses are given as the percentage of input cpm immunoprecipitated.
[b] Input cpm: 2.8×10^4.
[c] Input cpm: 1.8×10^5.
[d] Input cpm: 4×10^5.

The serum of guinea pigs immunized with EN antigens effectively precipitated both the viral EN and the HCMV antigens extracted from infected cells to the same extent as sera from guinea pigs immunized with WV (Table III).

Lymphocyte proliferation responses. The lymphocyte proliferation responses of guinea pigs immunized with soluble EN, NC, or virion antigens are shown in Table IV. The stimulation index was 3.6–54.3 times higher in cultures of lymphocytes from immunized animals (GP-A to GP-K) than in cultures of lymphocytes of nonimmunized animals (GP-L and GP-M), and there was no correlation between the degree of stimulation and the type of the antigen administered.

Discussion. An important approach to the prophylaxis of HCMV lies in the immunogenic analysis of the virus. In this study, the immunogenicity of separated HCMV NC and EN is described. We have used the surface-active detergent NP40 to dissolve the viral membrane and to dissociate the HCMV EN from the NC as demonstrated by electron microscopy. The results indicate that antigenically active material in sufficient quantity to elicit an antibody response can be obtained by treatment of HCMV with NP40. The data also show that antigens of the HCMV EN induce both humoral and cellular immunity comparable to that following inoculation of WV.

The role played by HCMV EN antigen in infections has not yet been shown. However, in the case of Herpes simplex virus, the results of previous studies suggest that the major function of the virion EN is to mediate entry of the NC into the host cell so that infection can be initiated. This assumption is based on findings that naked NCs are not infectious;

TABLE IV. LYMPHOCYTE PROLIFERATION RESPONSES TO CMV ANTIGENS IN IMMUNIZED GUINEA PIGS[a]

Guinea pig	Immunizing antigen	Stimulating antigen	Stimulation index
GP-A	Virion	Virion	13.4
		Envelope	4.9
GP-B	Nucleocapsid	Virion	18.7
GP-C[b]	Envelope	Virion	8.2
		Envelope	7.5
GP-F[b]	Envelope	CMV-cell extract	2.9
GP-G[b]	Envelope	CMV-cell extract	43.5
GP-H[b]	Envelope	CMV-cell extract	7.1
GP-K[b]	Envelope	CMV-cell extract	36.0
GP-L	None	CMV-cell extract	0.5
GP-M	None	Virion	0.7
		Envelope	0.8

[a] Cpm in CMV antigen-stimulated lymphocyte culture ÷ cpm in MRC-5 control antigen-stimulated culture.
[b] Guinea pigs received NP40-treated envelope antigen.

that neutralizing antibodies are directed against EN constituents; and that the absence of a particular glycoprotein from the virion EN results in the loss of specific infectivity (20). Thus, it is not surprising that the anti-EN antibody in the present study showed more neutralizing activity than the anti-NC antibody.

When complement-fixing and immunofluorescent antibodies were examined, the anti-EN antibody contained also antibody against cellular components. This might call into question the contamination of the antigen preparation with cellular proteins. Another possibility is that there are cellular proteins incorporated into the envelope of the virus, as it was demonstrated in the case of other budding virions (21). Recent reports indicate the insertion of cellular DNA sequences in the CMV genome which might be responsible for coding for viral proteins with cellular specificity (22). To elucidate these possibilities, further studies are necessary.

That the immunized guinea pig sera with NC stained the early nuclear antigen suggests that some of the early proteins are structural proteins and are incorporated into the nucleocapsid. The sera of virion-immunized guinea pig failed to stain the early nuclear antigen, suggesting that these early proteins are not present in the envelope, but in the deeper, nucleocapsid part of the virion. When virion preparations are used for immunization the immunogenic effect of these proteins is not enough to induce antibodies at the detectable level.

Guinea pigs immunized with NE antigens also developed a cellular immune response as demonstrated by the proliferation of their lymphocytes in the presence of CMV antigens. The importance of cellular immunity in preventing CMV infection has been suggested (4). Guinea pigs immunized with EN antigens developed both humoral and cellular immune responses that reached levels comparable to those in animals immunized with WV. Complete adjuvant in addition to the antigen was necessary to induce the antibody response.

As was previously shown by others (17), HCMV contains 19–32 polypeptides, most of which appear to be virus-specified. The polypeptides include 10–15 nonglycosylated species and about nine glycoproteins (17). Results obtained in this study indicate that there are 12 polypeptides (mol-wt range 50- to 120K) in the EN preparation, although we have not yet determined that they are all virus-coded.

Our data suggest that it may be possible to prepare a subunit CMV vaccine. Such a vaccine would contain neither live virus nor its DNA, thus ruling out the possibilities of oncogenicity or fetal transmission of CMV. More detailed analyses are needed to characterize the individual polypeptides contained within the EN fraction. The presence of cellular proteins in the viral antigen preparation is a problem requiring efforts toward further purification. On the other hand, since the proteins are syngeneic human proteins they may present no danger in actual use, as is the case for rubella and rabies vaccines.

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1. Weller TH. The cytomegalovirus: Ubiquitous agents with protean clinical manifestation. N Engl J Med **285**:203–214, 1971.
2. Plotkin SA, Farquhar J, Hornberger E. Clinical trials of immunization with Towne 125 strain of human cytomegalovirus. J Infect Dis **134**:470–475, 1976.
3. Plotkin SA, Friedman HM, Starr SE, Arbeter AM, Furukawa T, Fleisher GR. Prevention and treatment of Cytomegalovirus infection. In: Nahmias A, Dowdle WR, Schinazi RF, eds. The Human Herpes Viruses. New York, Elsevier, pp403–413, 1981.
4. Plotkin SA. Is it possible to vaccinate against herpes virus infections and their effects? Behring Inst Mitt **63**:123–134, 1979.
5. Albrecht T, Rapp F. Malignant transformation of hamster embryo fibroblasts following exposure to ultra-violet irradiated human cytomegalovirus. Virology **55**:53–61, 1973.
6. Huang ES, Roche JK. Cytomegalovirus DNA and adenocarcinoma of the colon: Evidence for latent viral infection. Lancet **1**:957–960, 1978.
7. Furukawa T, Fioretti A, Plotkin SA. Growth characteristics of cytomegalovirus in human fibroblasts with demonstration of protein synthesis early in viral replication. J Virol **11**:991–997, 1973.
8. Pritchett RF. DNA nucleotide sequence heterogenicity between the Towne and Ad169 strains of cytomegalovirus. J Virol **36**:152–161, 1980.
9. Wentworth BB, French L. Plaque assay of cytomegalovirus strains of human origin. Proc Soc Exp Biol Med **135**:253–258, 1970.
10. Jacobs JP, Jones CM, Belle JP. Characteristics of a

diploid cell designated MRC-5. Nature (London) **227**:168–173, 1970.

11. Forghani G, Schmidt NJ, Lennette ET. Antisera to human cytomegalovirus produced in hamsters. Reactivity in radioimmunoassay and other antibody assay systems. Infect Immun **14**:1184–1190, 1976.
12. Starr SE, Dalton B, Garrabraut T, Paucker K, Plotkin SA. Lymphocyte blastogenesis and interferon production in adult human leucocyte cultures stimulated with cytomegalovirus antigens. Infect Immun **30**:17–22, 1980.
13. Cremer NE, Schmidt NJ, Jensen F, Hoffman M, Oshiro LS, Lennette EH. Complement-fixing antibody in human sera reactive with viral and soluble antigens of cytomegalovirus. J Clin Microbiol **1**:262–267, 1975.
14. Waner JL, Weller TH, Stewart JA. Cytomegalovirus. In: Rose NR, Friedman M, eds. Manual of Clinical Immunology. Washington, D.C., Amer Soc for Microbiol, pp423–427, 1976.
15. Lowry OH, Rosenbrough NJ, Farr A, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem **193**:265–275, 1951.
16. Dulley RJ, Grieve PA. A simple technique for eliminating interference by detergents in the Lowry method of protein determination. Anal Biochem **64**:136–141, 1975.
17. Stinski MF. Human cytomegalovirus: Glycoprotein associated with virions and dense bodies. J Virol **19**:594–609, 1976.
18. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T-4. Nature (London) **227**:680–685, 1970.
19. Kessler SW. Rapid isolation of antigens from cells with a Staphylococcal protein A-antibody adsorbent: Parameter of the interaction of antibody–antigen complexes with protein A. J Immunol **115**:1617–1624, 1975.
20. Spear PG, Sarmento M. The structural proteins and glycoproteins of herpes viruses. In: The GE, Henle W, Rapp F, eds. Oncogenesis and Herpes Viruses III. Lyon, Intern Agency for Res on Cancer, p157, 1978.
21. Aupoix M, Vigier P. Evidence for a host cell surface antigen on the envelope of avian tumour viruses. J Gen Virol **27**:151–161, 1975.
22. Rüger R, Fleckenstein B. Homologies between the genome of human cytomegalovirus strain 169 and human cellular DNA. Conference on Pathogenesis and Prevention of Human Cytomegalovirus Infection, Philadelphia, Pa. April 20–22, 1983; CMV: Pathogenesis and Prevention of Human Infection, Birth Defects original articles series. New York, Liss, Vol 20, in press.

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