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Colburn Virus: Biological And Serological Characterization Of An Unusual Cytomegalovirus Isolate From Human Brain.

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COLBURN VIRUS: BIOLOGICAL AND SEROLOGICAL CHARACTERIZATION OF AN UNUSUAL CYTOMEGALOVIRUS ISOLATE FROM HUMAN BRAIN

by

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LIST OF ABBREVIATIONS

African green monkey	AGM
African green monkey kidney	GMK
Chick embryo	CE
Central nervous system	CNS
Colburn	Col
Complement fixation	CF
Continuous cell line derived from African green monkey kidney cells	BSC-1
Cytomegalovirus(es)	CMV(s)
Cytomegalovirus isolate from African green monkey	CSG
Cytopathic effect	CPE
Fluorescent antibody	FA
Hematoxylin and eosin	H and E
Herpes simplex virus	HSV
Human embryonic kidney	HEK
Human amnion	HA
Human fibroblasts	HF
Multiplicity of infection	MOI
Neutralization	NT
Phosphate buffered saline	PBS
Plaque forming unit	PFU
Rabbit kidney	RK

LIST OF ABBREVIATIONS (continued)

Varicella-Zoster	V-Z
5-iodo-2-deoxyuridine	IDU
50% cytopathic changes in tissue cultures	$TCID_{50}$

I. INTRODUCTION

Cytomegaloviruses (CMVs) are a ubiquitous group of agents which are known to cause latent infections in a wide spectrum of mammalian species. However, *in vivo*, CMV infections are considered strictly species specific and normally pose no serious threat to the adult hosts. CMVs are classified as members of the herpesvirus family on the basis of common physio-chemical and morphological properties. This group of agents is distinguished from other lytic herpesviruses by a slow replicative cycle, a distinctive cytopathology and a high degree of species specificity. This *in vitro* species specificity of human CMV remains valid, although not absolute, as human isolates productively infect only cells of human origin with few exceptions. On the other hand, the inverse relationship which implies that human cells are only permissive for human CMV is invalid since CMV from non-human primates replicate quite well in human fibroblasts.

In vivo, the species specificity of CMV remains valid as infection of a heterologous host has not been demonstrated for CMVs from any species.

The variability of human CMV regarding site of isolation and range of clinical manifestations has led to proposals that strains of human CMV may exist which differ in tissue tropism or pathogenic potency. Biologic comparisons of human CMVs have failed to detect significant markers to support such proposals. Limited serologic

heterogeneity has been reported among human strains, although these differences have not convincingly provided a method for subclassification, especially with regard to pathologic potential in man. Despite their antigenic similarity, as indicated by broad cross-reactivity, human CMV strains are thought to share only a nominal degree of antigenic homology with non-human strains.

The isolation of the Colburn (Col) virus presented an opportunity to characterize an agent with properties unique among the known herpesviruses. The recovery of a CMV-like agent from the brain of an immunocompetent child with no history of congenital acquisition had not previously been reported. However, regardless of any medical implications, the Col virus was of interest since it most closely resembled a CMV but differed from any other known human strain. Instead it shared certain biologic characteristics with simian CMV. These biologic similarities raised the possibility that Col virus was an unusual simian CMV which possessed pathogenic potential for man or may have originated as a contaminant of the isolation procedure. Characterization of the Col virus with simultaneous comparisons with human and simian CMV was important to determine if this agent was an unusual CMV or a new herpesvirus isolate.

II. LITERATURE REVIEW

Historical

The eventual emergence of CMV as an entity among the herpesviruses was a consequence of a convergence of knowledge from human and animal studies.

In humans, greatly enlarged cells with intranuclear and intracytoplasmic inclusions were first described over 70 years ago (Jesionek and Kiolemenoglow, 1904; quoted from Smith, 1959). These cells were observed in the lungs, liver and kidneys of a child thought to be syphilitic. Later, these types of cells were reported to be protozoan parasites and were classified as a species of Entamoebae (Smith and Weidman, 1910; quoted from Smith, 1959). The term "cytomegalic" was suggested to describe these inclusion-bearing cells, and the resemblance of these cells to those reported in the salivary glands of guinea pigs was noted by Goodpasture and Talbot (1921). The association between these cells and a viral agent was asserted when the synonomy between these cells and those previously described in herpetic lesions was recognized (Von Glahn and Pappenheimer, 1925).

In animals, experimental models were established and it was in such systems that the viral etiology of cytomegalic cells was proven. The cytomegalic cells in the parotid glands of guinea pigs were the first reported to be caused by a virus and this find-

ing was extended to other animal species where the strict species specificity of CMV was documented by Cole and Kuttner (1926) and Kuttner and Wang (1934). These same investigators considered the possibility that presence of similar cells found in humans might be due to a virus closely related to the salivary gland virus of animals; however, their attempts to propagate human CMV in animals were unsuccessful.

Smith (1954) undertook the *in vitro* propagation of murine CMV as a model for her subsequent attempts to isolate human CMV. Smith (1956) isolated and propagated human CMV from post-mortem salivary gland and kidney tissue of infants. In Boston, Weller *et al.* (1957) isolated CMV from three living infants suffering from cytomegalic inclusion disease (CID), one from a liver biopsy and two from urine specimens. Rowe *et al.* (1956), while recovering a new group of agents, the adenoviruses, also isolated human CMV from cultures of normal human adenoidal tissue.

The name cytomegalovirus was proposed by Weller, Hanshaw and Scott (1960) to include the large group of agents formerly called salivary gland viruses of animals and cytomegalic inclusion disease agents in man.

Clinical and Epidemiological

The most extensively studied clinical disease caused by CMV has been cytomegalic inclusion disease, a congenital infection passed to the fetus from an infected mother. The magnitude of CMV as a potential perinatal pathogen, accounting for 90% of all active

maternal infections, was reported by Alford et al. (1974). Among infants with overt cytomegalic inclusion disease, a spectrum of clinical abnormalities was observed which testified to the ability of CMV to infect virtually any organ system, the central nervous system being one of the more vulnerable. Microcephaly, mental retardation and cerebral palsy have been observed in most infants having the congenital form of the infection (Hanshaw et al., 1964).

CMV infections occur with inordinate frequency in immunosuppressed patients. Craighead, Hanshaw and Carpenter (1967) reported the development of CMV infections in 60% of renal allograft recipients. Another patient group with an increased incidence of CMV infections is that with generalized neoplasia. Sullivan et al. (1968) documented a high sero conversion rate in leukemic patients originally lacking CMV antibody.

CMV also causes disease in immunocompetent hosts. CMV-mononucleosis is the term used to describe a syndrome similar to the more common infectious mononucleosis; however the two are clinically separated by the heterophile antibody test which is negative in the CMV induced disease. The long-sustained infection of circulating leukocytes was a prominent mechanism in the transmission of this disease (Klemola et al., 1970).

The postperfusion syndrome, a disease similar to cytomegalic inclusion disease which occurs in some adults following multiple whole blood transfusion, is also related to the capacity of CMV to infect leukocytes. The association between CMV and this syndrome was confirmed by isolating the virus from leukocytes and demonstrating a

rise in antibody to CMV following transfusions (Lang, Scolnick and Wilkerson, 1968).

Acquired infection with CMV is extremely common with estimates as high as 80% of the population having antibody to CMV. The prevalence of CMV is dependent on several factors, age being the primary determinant. Rowe et al. (1956) found antibody to CMV in 14% of sera from infants 6-23 months of age, in 53% of specimens from young adults up to 25 years of age and in 81% from persons over 35 years of age. In general, postnatally acquired CMV infections produce no recognizable disease but remain latent unless stimulated by events which remain poorly defined.

Properties of the Virus

The physio-chemical properties of CMV are presented in Table 1, and are common to all herpesviruses. The presence of double stranded DNA as the genetic material of CMV has been demonstrated by a variety of cytochemical techniques and by the susceptibility of CMV to antagonists of DNA synthesis such as halogenated pyrimidines and deoxynucleoside analogues (Goodheart, Filbert and McAllister, 1963). The DNA from human CMV was first extracted in 1964 and reported to have a minimal molecular weight of 32×10^6 daltons. Equilibrium density gradient centrifugation indicated a guanidine plus cytosine content of 58% (Crawford and Lee, 1964). More recent studies using purified viral DNA reported both human and simian CMV co-sedimented in the region of 55S in neutral sucrose gradients; thus the molecular weights of these CMV DNAs was esti-

Table 1

Physical and Chemical Properties of Cytomegaloviruses

Nucleic acid	double stranded DNA
Symmetry	icosahedral
Number of capsomeres	162
Size of particle	140-180 mu
Size of nucleoid	45-75 mu
Lipid envelope	present
Sensitivity to ether	positive
Sensitivity to low pH	positive
Thermostability	heat labile at 56°C

mated as 100 x 10^6 daltons. The density of viral DNA was 1.716 g/cm^3 for human strains and 1.710 g/cm^3 for the simian strain (Huang, Chen and Pagano, 1973).

The buoyant density values for human and simian CMV DNAs have been verified by other investigators who examined 28 herpesviruses, including CMV of human, simian and murine origin, in an attempt to subgroup these agents on the basis of DNA density. However the CMVs did not form a subgroup apart from the other herpesviruses (Goodheart and Plummer, 1975). DNA of human CMV has been examined for homology to other herpesviruses including simian CMV, and no relatedness or less than 5% homology was detectable (Huang and Pagano, 1974). The size, symmetry and number of capsomers of CMV were determined by electron microscopy. Although these techniques have been useful taxonomic tools for segregating herpesviruses from other virus families, they were unable to discriminate CMV from other herpesviruses (Wright, Goodheart and Lielausis, 1964; Becker, Melnick and Mayor, 1965).

CMV is readily inactivated by exposure to ether, chloroform and other lipid solvents or when kept at a pH below 5.0 (Rowe et al., 1956; Hamparian, Hilleman and Ketler, 1963). Infectivity is also destroyed by exposure to 56°C for 30 minutes or 4°C for 1 week; however CMV can be stored in the presence of a stabilizer, e.g., serum or sorbitol, at -70°C or in a liquid nitrogen freezer.

In Vitro Replication

Two distinctive properties in the replicative cycle of CMV

are its slow growth rate and the tendency of progeny virus to remain cell-associated. The chronology, site of replication and additional properties related to CMV replication are presented in Table 2. CMV is contrasted to Herpes Simplex Virus (HSV), the prototype herpesvirus, to illustrate the distinguishing characteristics of CMV.

The replicative cycle of CMV required approximately 4 days whereas HSV completed its cycle in 8 hours. Smith and DeHarven (1974) have shown absorption of CMV occurred at 4° and penetration occurred within 3 minutes after the temperature was raised to 37°. Once CMV entered the cell, it migrated to the outer aspect of the nuclear membrane but remained relatively intact for periods up to 48 hours before uncoating began. This lag between penetration and uncoating appeared to be a major contributory factor to the lengthy replication of CMV although ensuing events also appeared retarded in comparison to HSV. Viral DNA replication was confined to intranuclear sites analogous to the inclusion. It was determined that viral DNA, which appeared later in the cytoplasm, was not synthesized there but was transferred from the nucleus, probably in the form of complete virions (Goodheart, McAllister and Filbert, 1964).

Transcription of viral mRNA preceded DNA replication thus indicating some early proteins were coded for by the parental DNA, however, the bulk of mRNA and protein synthesis followed DNA replication (Furukawa, Fioretti and Plotkin, 1973). The structural proteins synthesized in the cytoplasm migrate into the nucleus where new virions were assembled.

CMV differed from HSV regarding the effect of viral repli-

Table 2

Replicative Events of Cytomegalovirus (CMV) and Herpes Simplex Virus (HSV)

Event	Time of Maximal Activity # CMV	HSV
Penetration	3 minutes	3 minutes
Uncoating	6-24 hours	1-2 hours
Protein synthesis in cytoplasm	12-24 hours	4-6 hours
DNA synthesis in nucleus	24-72 hours	4-6 hours
Assembly of virions in nucleus	24-96 hours	6 hours
Envelopment and release	48-96 hours	8 hours
Cell lysis	7-8 days	1-2 days
	Other Replicative Properties	
Inclusions	intranuclear and intracytoplasmic	intranuclear
Amount of infectious extracellular virus	10^6 PFU/ml*	10^8 PFU/ml
Effect on host cell macromolecular synthesis	stimulation	total inhibition

*Highly adapted strains of CMV

#Times expressed vary with virus input and cell system

cation on host cell macromolecular synthesis. Infection by HSV resulted in a rapid cessation of host DNA, RNA and protein synthesis whereas CMV infection did not inhibit these processes. Host cell DNA and RNA synthesis in contrast, were stimulated by CMV infection as evidenced by an increased mitotic index and increased incorporation of tritiated thymidine and uridine (St. Jeor et al., 1974; Furukawa, Tanaka and Plotkin, 1975).

The virus assembled in the nucleus acquired an envelope at the inner layer of the nuclear membrane as the particles passed into the space between the inner and outer layers of this structure. Unenveloped particles were sometimes observed in the cytoplasm and these particles appeared to acquire their envelopes by budding into cytoplasmic vacuoles. The release of infectious virus normally occurred by cell lysis around the seventh day post infection (Smith and DeHarven, 1973).

The majority of studies referenced above used CMVs which had been serially passaged multiple times, thus were highly adapted to in vitro growth. Newly isolated CMV strains required much longer incubation periods to produce CPE equivalent to adapted strains. This was due to the high degree of cell-association of progeny virus which was gradually overcome by repeated passage. The amount of infectious extracellular virus released from infected cells appeared to reflect the degree of adaptation, thus the amount of extracellular virus presented for CMV in Table 2 approached the maximum titer obtainable with adapted virus and greatly exceeded those normally obtained with a wild type CMV. Kanick and Craighead (1972) have

reported differences in the replicative events of wild and adapted strains of CMV; however the exact effects of prolonged in vitro growth on CMV remain obscure.

CMV, like other herpesviruses, appeared to replicate very inefficiently in vitro as witnessed by the accumulation of large numbers of incomplete or empty capsids. The infectious unit to virus particle ratio for CMV has been estimated to be as low as 1 PFU per 10^6-10^8 physical particles, however a later report increased the ratio to 1 PFU per 10^2-10^3 (Smith and Rasmessen, 1963; Benyesh-Melnick et al., 1966).

Cytopathology of CMV Infected Cells

A distinctive property, considered to be pathognomonic for CMV, is the cytopathology of infected cells which is characterized by production of both intranuclear and intracytoplasmic inclusions. Initial cytopathic effects (CPE) are focal, with the appearance of small collections of swollen rounded translucent cells. The individual foci increase slowly in size as the adjacent cells became involved and there is a concomitant degeneration of the central area. Examination of affected cells after hematoxylin and eosin staining reveals distorted enlarged nuclei, each containing one or more large sausage-shaped, eosinophilic inclusion bodies. The inclusions occupy most of the nuclear area, being separated from the nuclear membrane by a narrow clear zone or halo. There are generally one or two densely basophilic bodies, presumably nucleoli, on the nuclear membrane or in the halo zone. This halo separates the inclusion from

the marginated chromatin also located on the nuclear membrane. Often eosinophilic or amphophilic cytoplasmic inclusions are found adjacent to the concave surface of the nuclear inclusion (McGavran and Smith, 1965; McAllister et al., 1963).

The nuclear inclusion is the site of viral DNA replication and virus assembly, whereas the cytoplasmic inclusions appear to be aggregates of mature virus particles which have migrated into these vacuoles. Several morphological types of such vacuoles have been described by EM studies with the larger types being seen as cytoplasmic inclusions by light microscopy. Virus particles found in these cytoplasmic inclusions were surrounded by large masses of non-viral material which appeared to be produced by microtubular membranes and the Golgi apparatus (Craighead, Kanick and Almeida, 1972; Iwasaki et al., 1973).

Host Cell Specificity

In vivo, CMV has not been shown to cross the species barrier or cause any disease in heterologous species; in addition, CMV appears to replicate primarily in the epithelial cells of its natural host. Conversely, in vitro the strict species specificity of CMV is not valid and replication is primarily, if not exclusively, in fibroblastic cell types.

Human CMV replicates most efficiently in human fibroblastic cell cultures, e.g., WI-38 or human skin and muscle, but has been isolated in HEK (Benyesh-Melnick, Rosenberg and Watson, 1964). Human CMV has also been reported to replicate in simian, bovine and rodent

tissue cultures, although the infections appeared to be either abortive or if productive, highly modified (Fioretti et al., 1973; MacFarlane and Sommerville, 1969; Waner and Weller, 1974).

Human fibroblastic cell cultures allowed replication of an adapted murine CMV and were also abortively infected when inoculated with murine CMV from homologous tissue (Kim and Carp, 1972; Raynard, Atanasiu and Virat, 1972; quoted from Weller, 1970). Human fibroblasts were permissive for two CMV isolates from simian species, African green and Rhesus monkeys. The CSG strain of CMV was isolated from salivary gland tissue of an African green monkey dying of unknown causes. In early passages, progression of CPE appeared after 22 days and was slow, requiring 6 weeks for complete destruction, but by the fourth passage, the incubation period to first CPE was reduced to 1-2 days with complete destruction in 10-14 days. The amount of extracellular virus produced by the agent was 10^5 $TCID_{50}$ per 1.0 ml at this time (Black, Hartley and Rowe, 1963). African green monkey CMV replicates well in human and simian fibroblasts, but does not replicate in rabbit kidney cells (Plummer et al., 1969). The Rhesus monkey CMV, 68-1 was isolated from urine of a healthy animal and also grew well in human fibroblasts but not in GMK or RK cells (Asher et al., 1974).

These data indicate that CMVs are by no means completely species specific for the cell cultures of their host species; on the other hand, these agents do not grow so readily in such a wide spectrum of host cells as do the other herpesviruses.

Serologic Relationships

Among the lytic herpesviruses of man, CMV has not been shown to share any major antigenic determinants with another member of the herpesvirus family (Krech and Jung, 1971).

The detection of antigenic heterogeneity among human CMV isolates has varied according to serologic method and type of antisera. The most widely used test for detecting CMV antibody has been the complement fixation (CF) test. This test has been successfully utilized in diagnostic procedures where it has been shown to be specific for CMV; however it has not been able to distinguish among human CMV isolates. Since human CMV are considered to share a mosaic of CF antigens, the AD169 strain has been the standard source of antigen used in this test (Starr, Califiore, and Casey, 1967; Benyesh-Melnick et al., 1965). Recently, a glycine buffer extraction method for antigen preparation has significantly increased the sensitivity of the CF test; thus, future studies may reveal antigenic diversity among human CMVs by this method (Krech, Jung and Sonnebend, 1971; Cremer et al., 1975).

The fluorescent antibody (FA) test has been utilized to diagnose CMV infection and to quantitate the amount of CMV in some procedures (Rapp, Rasmessen and Benyesh-Melnick, 1963; Waner and Budnick, 1973). The indirect FA test has been shown to be specific for CMV antibodies and has the added advantage of defining both the class of human antibody and the location of the antigen in or on the infected cell (The and Langenhuysen, 1972; The, Klein and Langenhuysen, 1973). The FA test has also proven to be the most sensitive sero-

logic technique for the demonstration of CMV antibody (Spencer and Andersen, 1972). Some antigenic differences have been reported using the FA test (Gonczol and Andersen, 1973).

The neutralization test (NT), considered the most specific test for CMV, is the least sensitive of these methods. The original demonstration of antigenic diversity among human CMVs was reported using this test and on the basis of those results, it was proposed that CMVs be tentatively designated as three serotypes (Weller, Hanshaw and Scott, 1960). This test has subsequently been employed by other investigators with results indicating varying degrees of antigenic heterogeneity among these agents (Andersen, 1970, 1971, 1972ab; Plummer and Benyesh-Melnick, 1964).

These same procedures have been used to examine the relatedness of human and simian CMV since these are the only CMV strains which cannot be differentiated on the criteria of growth in human tissues. The CF test revealed a one-way cross-reaction; sera from simians fixed complement in the presence of human CMV, while human sera had no CF activity for monkey CMV (Black, Hartley and Rowe, 1963; Dreesman and Benyesh-Melnick, 1967).

The FA test has not demonstrated any intraspecies cross-reactivity between human and simian CMV (Chiang, Wentworth and Alexander, 1970; Huang, Huang and Pagano, 1974).

The neutralization test has been utilized more frequently for the studies dealing with relatedness between human and simian CMV and has also been species specific when hyperimmune sera were employed, although one-way cross-reactivity was demonstrated with

sera from naturally infected monkeys having neutralized a human CMV Minamishima, Graham and Benyesh-Melnick, 1971; Graham et al., 1971).

III. MATERIALS AND METHODS

Source and Processing of Clinical Specimens

The Colburn virus was isolated from brain tissue obtained by biopsy from a patient whose clinical history is summarized here.

Three weeks prior to hospital admission (Children's Hospital, Birmingham, Alabama) of this 6-year-old male, Diphtheria-Pertussis-Tetanus immunization was administered. Two days later, he began to experience an unsteady gait, slurring of speech, poor vision and lethargy, all of which progressed up to the time of admission. Results of neurological and laboratory tests indicated severe brain damage. Based on these findings and a recent history of a disease compatable with herpes simplex gingivostomatitis, a brain biopsy was performed on the ninth day of hospitalization. Pathologic examination of the brain tissue revealed only one intranuclear inclusion-bearing cell and no evidence of inflammation, cellular infiltrates or hemorrhagic necrosis. Virologic examinations of throat swab material, urine, white blood cells and spinal fluid were repeatedly negative throughout the hospital course. These findings prompted a diagnosis of encephalopathy for which no toxic cause could be found.

The brain tissue was finely minced in complete growth media and 0.2 ml inoculated as a 20% suspension. The suspension was stored at -70°C in 10% dimethyl sulfoxide. Fecal and throat swab materials

were rinsed in 2 ml of Hanks' balanced salt solution containing 25,000 U penicillin, 25,000 ug each of streptomycin and neomycin and 250 units of bacitracin per ml. The resultant suspension was clarified and inoculated in 0.2 ml amounts. Urine and spinal fluid were inoculated in the same amounts without treatment. All inoculated cultures and controls were microscopically examined at two-three day intervals for three-six weeks and cells and supernate passed at least once with similar observations before being discarded as negative.

Tissue Cultures and Media

Monolayer cultures of the following primary cell types were employed: Human Embryonic Kidney (HEK), African Green Monkey Kidney (GMK), Human Amnion (HA), Chick Embryo (CE) and Rabbit Kidney (RK). The former 3 were purchased as roller tube cultures from Flow Laboratories (Rockville, Md.) and the latter two were prepared in our laboratory. Continuous cell lines, carried in our laboratory, included: African Green monkey kidney (BSC-1), HeLa and Hep-2. Human fibroblasts employed were discontinuous cell lines usually utilized between passages 1-30. They originated from human fetal lung or foreskin tissue collected from newborns. These cell types were used interchangeably and are collectively referred to as HF cells.

All roller tube cultures prepared in our laboratory employed Minimal Essential Medium with Hanks' salts as the growth medium. Upon forming confluent monolayers, or when purchases as such, cells were sustained with Medium 199 with Earle's salts as the main-

tainance media. Growth media was supplemented with 10% newborn calf serum whereas the maintainance media utilized 5% serum and both received Gentamicin (50 ug/ml) and l-glutamine (2 mM).

Human and Simian Strains

The two prototype human CMVs, AD169 and Davis have been passaged in our laboratory for several years since their acquisition from Dr. T. H. Weller's laboratory (Harvard School of Public Health, Boston, Mass). Both these agents have been extensively passaged in tissue culture since their original isolation and are thus highly adapted to tissue culture (Rowe et al., 1956; Weller et al., 1957). The CSG strain isolated from African Green monkey was purchased from the American Type Culture Collection, Rockville, Md. This agent was used during the 20th to 38th passage in our laboratory following over 300 consecutive passages in human fibroblasts prior to acquisition (Black, Hartley and Rowe, 1963). The 68-1 strain from Rhesus monkey urine was purchased from the same source and employed between the 10th and 15th passages in our laboratory (Asher et al., 1974).

Virus Passage and Storage

Clarified supernates from infected cell cultures were utilized as inocula when seeking extracellular virus whereas scraped cells plus supernate were inoculated to detect cell associated as well as extracellular virus. Viral agents were passaged by one of the above methods when 80-90% of the cell monolayer displayed CPE.

Virus stock pools of each agent were prepared as follows and kept at -70°C in a Revco freezer. Infected monolayers showing

80-90% CPE were washed once with phosphate buffered saline (PBS) and scraped into PBS and centrifuged. The cell pellet was resuspended in Tris buffer (pH 7.6) and sonicated for 5 minutes at 25 watts with a microtip sonifier to disrupt the cells. Following clarification by low speed centrifugation, sorbitol was added to a final concentration of 10% and 0.5 ml amounts of the pool aliquoted into screw cap vials for storage. Stock pools prepared in this manner were titrated prior to and during storage to insure that the infectivity remained stable. Stock pools were utilized within 3 months during which time the infectivity titer remained stable.

Viral Quantifications

Quantitation of infectious virus was performed by either a microplaque assay system or by roller tube titrations in HF cell cultures. Roller tube titrations used serial 10-fold dilutions of virus containing inocula and three tubes per dilution. Tubes were inoculated with 0.2 ml quantities and allowed to adsorb for a minimum of 24 hours prior to changing of media. The tubes were observed for two weeks before $TCID_{50}$ end point determinations were calculated by the method of Reed and Muench (1938). Plaque assays were done in 24-well Disposo-Trays (Linbro Plastics) using serial 10-fold dilutions. Following inoculation of 0.05 ml into duplicate wells, the inocula were adsorbed for 1 hour, removed and replaced with a semi-solid overlay composed of: minimal essential medium with Earle's salts, 0.0017% sodium bicarbonate, 5% calf serum and 2.5% methyl cellulose. The overlayed HF monolayers were observed until distinct

plaques were visible, usually one week for Col, CSG and Davis and 2 weeks for AD169 and 68-1. Monolayers were fixed by addition of 10% formalin, both fixative and overlay aspirated after cooling to liquify the methyl cellulose, and stained for 30 minutes with 0.03% methylene blue. Stain was removed; monolayers rinsed with water, and plaques counted with titers expressed as PFU per 1.0 ml.

Suppression of Colburn with 5-iodo-2'-deoxyuridine

Monolayer cultures of HF cells were pretreated for 24 hours with either 25 or 50 μg/ml of 5-iodo-2'-deoxyuridine (IDU purchased from Nutritional Biochemical Corporation, Cleveland, Ohio). Stock viral harvests were simultaneously titrated in the treated and untreated control cultures. The cultures were observed for two weeks and the log suppression of virus calculated. AD169 served as the DNA control virus and Vesicular Stomatitis Virus as the known RNA virus.

Ether and Acid Tests

Diethyl ether was mixed with viruses in a proportion of 1 part ether to 4 parts extracellular virus suspension and incubated in a closed container for 24 hours. Ether was removed by evaporation until no fumes were detectable, approximately 10 minutes, and 10-fold dilutions of the suspension were titrated by the roller tube method. Herpesvirus hominis, a known ether sensitive virus, and Type 12 Adenovirus, a known ether resistant agent were handled in an identical manner. An equal volume of each virus was also titrated without ether treatment and infectivity of the viruses incubated with and

without ether were compared.

Acid stability tests used 1.0 ml amounts of minimal essential media containing extracellular viruses. The pH was lowered to approximately 3.0 by the addition of 0.05 ml of 1 N hydrochloric acid and the medium was incubated for 3 hours at room temperature. The pH was raised to neutrality by adding 0.05 ml of 1 N sodium hydroxide, and viruses were titrated by the roller tube method. AD169 was used as the positive control while $ECHO_{11}$, which was titrated in BSC-1 cells served as the negative control. Untreated aliquots of each virus were also titrated in an identical manner.

Comparative Growth Studies

For comparative growth studies, virus stock pools were adjusted to yield a multiplicity of infection (MOI) of approximately 1 PFU per cell. Growth curves were constructed by monitoring the extracellular virus production at 24-hour intervals from pooled supernates of three infected roller tube cultures. All comparative growth studies were done simultaneously in each cell system and the microplaque technique was used for quantitation of virus in HF cells.

For thymidine uptake studies, HF cell cultures grown in 60 x 15 mm plastic petri dishes (Falcon) were utilized. Following a one-hour period for virus adsorption, cells were washed with PBS and maintainance medium containing 2.5 μC of thymidine-methyl-H^3 (specific activity 6 μC/m mole, Schwartz-Mann) was added. At the specified intervals, media was removed, and cells were harvested by scraping into chilled PBS. Thymidine incorporation was assayed by the

procedure of Cohen, Vaughn and Lawrence (1971) in a Packard Tri-Carb liquid scintillation spectrometer. These studies were carried out in collaboration with Dr. Leigh Charmella.

Animal Studies

Litters of 3-day old, albino, Swiss mice were randomly assorted into 15 groups of 14 mice according to route of administration which included intramuscular, intraperitoneal and intracranial. 5 x 105 TCID of Col, AD169 and Davis viruses grown in HF cells were inoculated. Controls included animals inoculated with medium from uninfected cell cultures as well as uninoculated animals.

Comparative Serial Passage and Cytopathology

Initially, equal MOI of approximately one PFU per cell of each viral strain was employed as a 0.2 ml inoculum. Thereafter, viruses were serially passaged, in the same amounts, when CPE reached 80-90%; no attempt was made to equalize MOI on serial passage because of differences in cytopathic and growth potential between agents. Extracellular virus production was employed as the comparative monitor for cell adaptability and quantitation was performed in HF cells with three tubes of cell culture per 10-fold dilution by the roller tube method.

If no CPE developed within 2 weeks following inoculation, cells were stained with H and E to assess for abortive infection. If CPE was observed in stained preparations, scraped cells and supernatant fluids from the same batch of infected cultures were transferred to permissive HF cells and observed for CPE for 1 month before

being discarded as negative. When CPE developed slowly, serial passage was accomplished with scraped cells and supernates to allow for propagation of intracellular virus. Otherwise, only centrifuged supernatant fluids were utilized for serial passage. The same cell batch was used for propagation of viruses at each passage level.

For comparison of the development of CPE and plaque formation, a low multiplicity of infection (0.1 PFU/cell) was used. Cytopathic development was monitored in roller tube cultures with fluid overlay, while plaque morphology was observed in slanted stationary cultures under an overlay with 5% methyl cellulose. Cell changes were microscopically examined in living cells at daily intervals and also, in fixed preparations (Bouin's) stained with H and E.

Preparation and Source of Antisera

Goat and primate hyperimmune sera prepared against human strain AD169 or the simian strain G-2757 from African Green Monkey were the gifts of the Reference Reagents Branch of the National Institute of Allergy and Infectious Diseases. The serologic reactivities of these sera have been previously described (Haines, Von Essen and Benyesh-Melnick, 1971; Graham et. al., 1971). Rabbit hyperimmune sera prepared against Davis and the Col virus at the Harvard School of Public Health, Boston, Mass. were a gift from Dr. Joseph Waner. The immunogens used for their production were glycine extracts from cells infected with the respective agents. Rabbit hyperimmune sera were also prepared in our laboratory by a modification of the method of Martos et al., 1970. Briefly, eleven New Zealand White rabbits

were pre-bled and divided into 4 groups. Three rabbits received AD169, Davis or Col virus; the 2 remaining animals which served as controls were injected with supernate from uninfected HF cells. Immunization consisted of 7 bi-weekly injections of 1.0 ml of freshly prepared extracellular virus or control material given intravenously plus 2.4 ml of a mixture of 2 parts virus or control and 1 part Freund's incomplete adjuvant given intramuscularly. The viral inocula had a minimum infectivity of 10^5 $TCID_{50}$ per 0.2 ml. Animals were bled intermittently after the fourth immunization and sera stored at -20°C.

Human sera were obtained from 2 groups of patients: pregnant and/or post partum women and their infants under study for perinatal CMV infections (Reynolds et al., 1973) or renal transplant patients with proven CMV infections. Sera collected from laboratory personnel were also employed.

Neutralization Tests

Neutralization was performed by a method similar to that of Haines et al. (1971). Sera were inactivated at 56°C for 30 minutes. Serial two-fold dilutions were then prepared in minimal essential medium without serum and mixed with equal parts of virus stock pools diluted in the same medium to contain approximately 1000 PFU per 0.2 ml. Guinea pig complement was added to the virus-serum mixtures to a final concentration of 5%. Then the mixture was incubated for 1 hour at 37°C prior to plaquing by a micro technique. Comparative studies were done simultaneously and control sera were always run concomitantly. Neutralization end points were expressed as the highest

final dilution producing a 60% reduction of the input virus.

Indirect Fluorescent Antibody Test

Antigen preparations used in the indirect Fluorescent antibody (FA) test were prepared by mixing equal parts of infected and uninfected HF cells grown in 250 ml flasks (Falcon Plastics, Oxnard, Ca.). The uninfected cells served as internal controls. Infected monolayers showing 70-80% CPE and uninfected cells were washed once with phosphate buffered saline (PBS) and removed from the flasks after brief incubation with equal parts EDTA (0.03% w/v) and trypsin (0.25% w/v). Cells were washed 2 times in PBS and diluted to a concentration of 3-5 x 10^5 cells per ml. One drop from a microtiter pipette (0.025 ml) of the cell admixture was placed within each of eight 6 mm circles on a printed slide (Carson Scientific Inc., Matteson, Ill.), air dried, fixed in cold acetone for 10 minutes, air dried again and stored at -70°C.

Antiserum to human IgG was raised in goats, absorbed and purified on solid immunoabsorbent columns, and conjugated with fluorescein isothiocyanate, in collaboration with Dr. Alexander Lawton. The conjugate had a molar fluorescein to protein ratio of 3.0 and elicited negligible non-specific staining on infected or uninfected cells. A 1:75 dilution (v/v) of 1.5 mg/ml of this conjugate was used in all studies.

Before staining, slides were brought to room temperature, then washed with PBS, and once again air dried. Single drops of serial, two-fold dilutions of sera were placed in appropriate wells and incubated for 1 hour at 37°C. Residual serum was removed by

washing twice with PBS, one drop of conjugate was added and the slides then incubated as above. After rinsing 3 times in PBS, coverslips were mounted on slides with 50% glycerol in PBS. Slides were read using a Leitz fluorescent microscope with excitation filters BG12 and BG38 and barrier filters K510 and K530. Titers were expressed as the reciprocal of the highest serum dilution which produced weak specific fluorescence. Positive (titer 1:256) and negative serum, as well as uninfected cellular controls, were included in each run and all comparative tests were performed simultaneously.

IV. RESULTS

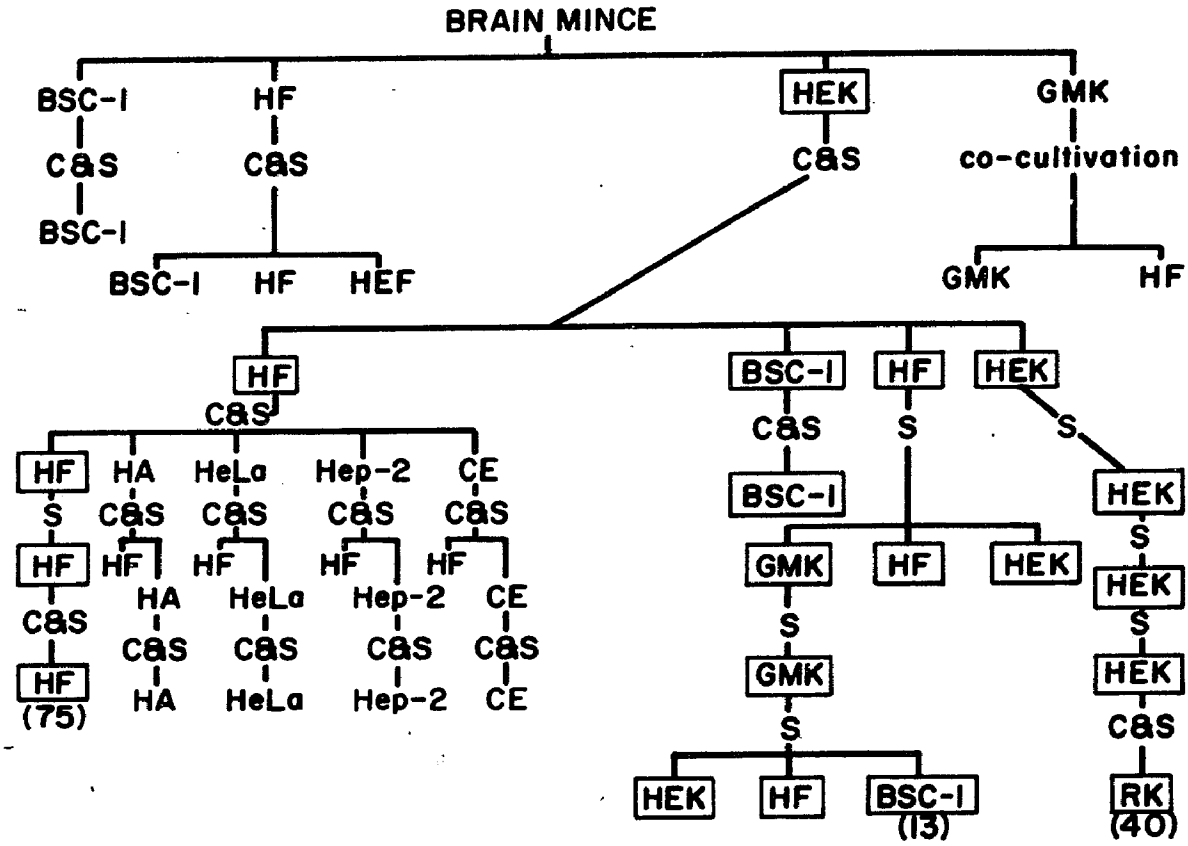
Classification of Col as a CMV

Isolation and Propagation

The isolation, re-isolation and early passage history of the Col agent are schematically represented in Figure 1. As depicted in the upper portion of the figure, the brain mince was inoculated into HEK, BSC-1, HF and GMK. No CPE was observed in HF in the original cultures nor when scraped cells and supernate from these were inoculated after 35 days into BSC-1, HF and HEK. The original GMK was co-cultivated 21 days following inoculation with homologous cells and HF; CPE failed to develop over a 3-week observation period. As seen in Figure 2, the HEK cells in one culture became swollen, rounded and refractile in areas immediately adjacent to the implanted brain mince 3 days after inoculation and similar CPE appeared in the other inoculated HEK cultures during the first week. The focal CPE progressed slowly to involve 90% of the HEK monolayers over a 4-week interval. Control cultures of HEK from the same batch of cells remained negative throughout four serial passages over a 6 week interval.

A few foci similar to those seen in HEK also appeared in the BSC-1 cultures but not until 27 days following inoculation at which time the cells were senescent. CPE failed to develop after passage of cells and supernate from these cultures back into BSC-1.

Figure 1. Isolation and re-isolation of Col virus. The following cell cultures were employed: Human Embryonic kidney (HEK), Human fibroblasts (HF), Human Amnion (HA), Chick Embryo (CE), Green Monkey Kidney (GMK), BSC-1, HeLa, Hep-2. Those cultures which displayed CPE are enclosed in solid lines. Serial passage employing cells and supernate (C&S) or supernate only (S) are indicated and the numbers enclosed in parenthesis indicate the number of serial passages in the various cell cultures. The original isolation history is depicted in the top panel and re-isolation data is shown at the bottom.


BRAIN MINCE
BSC-1
cas
BSC-1
HF
cas
BSC-1 HF HEF
HEK
cas
GMK
co-cultivation
GMK HF
HF
cas
HF
S
HF
cas
HF (75)
HA HeLa Hep-2 CE
cas cas cas cas
HF HF HF HF
HA HeLa Hep-2 CE
cas cas cas cas
HA HeLa Hep-2 CE
BSC-1
cas
BSC-1
HF
S
HEK
S
GMK
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S
HEK HF BSC-1 (13)
HF HEK
S
HEK
S
HEK
S
HEK
cas
RK (40)

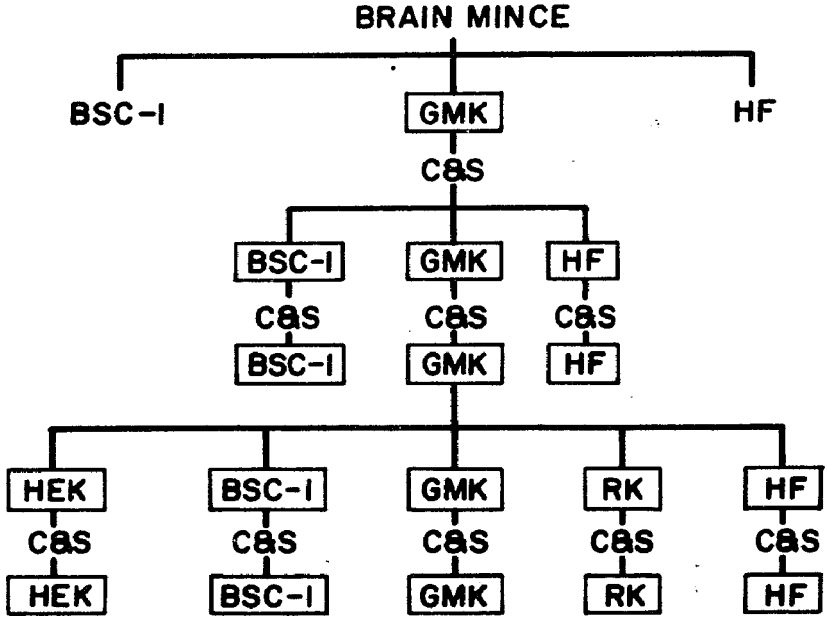

BRAIN MINCE
BSC-1
GMK
HF
cas
BSC-1 GMK HF
cas cas cas
BSC-1 GMK HF
HEK BSC-1 GMK RK HF
cas cas cas cas cas
HEK BSC-1 GMK RK HF

Figure 2. Early Col induced cytopathic changes seen in the original isolation. The dense mass of tissue in the upper portion is the minced brain growing on human embryonic kidney cells. The enlarged, rounded cells are present in areas immediately adjacent to the brain (unstained preparation, 100 x).

Scraped cells and supernate obtained at 3 days from the first positive cultures of HEK were inoculated in HF, HEK and BSC-1. Focal CPE developed in each cell system at 2, 6 and 14 days post-inoculation, respectively. Complete destruction of HF and BSC-1 occurred at days 14 and 21 while CPE advanced much more slowly in HEK.

The release of extracellular virus during early passages was confirmed by passing centrifuged supernate from the first passage of Col virus in HF cells into HEK, GMK and HF cultures. Quantitation of extracellular virus after 3 serial passages in HF cells showed low amounts of virus, 50 $TCID_{50}$/0.2 ml, were being released. After six passages, total virus production remained at low levels ranging from 10-100 $TCID_{50}$/0.2 ml, in HF cells. During these early passages, productive infection of human epithelial cell cultures, heteroploid cell lines or chick cells could not be established.

The re-isolation of Col virus was accomplished six months later from the original brain mince and is shown in the lower portion of Figure 1. Thawed brain mince was inoculated into BSC-1, GMK and HF; HEK tissue was unavailable at this time. Focal CPE developed only in the inoculated GMK, first appearing at 7 days. Initial foci were again sparse and confined to tissue adjacent to the brain mince. Cytopathology progressed slowly but the infection could be transferred to HF and BSC-1 by 14 days. It was noteworthy that the uninoculated GMK control cells remained negative for 30 days and no virus was recovered from them following co-cultivation with permissive HF cells or on serial passage of cells and supernate. The virus recovered

upon re-isolation appeared identical to the original isolate regarding host cell range, cytopathology in living cells and appearance of infected cells in stained preparations.

Physio-chemical Studies

The amount of suppression by 5-iodo-2-deoxyuridine (IDU) is presented in Table 3. The decrease in infectious virus resultant in IDU treated cultures compared to control cultures reflects the amount of suppression.

Table 3

Suppression of Col Virus Growth by 5-iodo-2-deoxyuridine

Virus	Control Titer	Titer with 25 ug IDU	Titer with 50 ug IDU	Log Suppression
Colburn	7.0*	1.5	1.5	5.5
AD169	7.0	3.0	3.0	4.0
VSV	7.0	7.0	7.0	0

*Titer of infectious extracellular virus expressed as $TCID_{50}$ per 1.0 ml.

The growth of Col virus was equally inhibited by 25 or 50 ug of drug. The positive control, AD169 was also markedly inhibited by both drug concentrations while the RNA virus, Vesicular Stomatitis Virus (VSV) was uneffected.

Col virus was compared to $ECHO_{11}$ to determine its stability at pH 3.0. Infectivity of Col was reduced by a factor of 6 logs,

$10^{6.5}$ to $10^{0.5}$ $TCID_{50}$ per 1.0 ml. $ECHO_{11}$, a pH-stable picornavirus, serving as the control, showed no decrease in titer.

Exposure of extracellular Col virus to diethyl ether reduced its titer by a factor of 5 logs, while identically treated ether stable Adenovirus controls remained unchanged.

Infectivity of Col virus was totally destroyed by heating to 56°C for 30 minutes.

Inclusion Body Formation

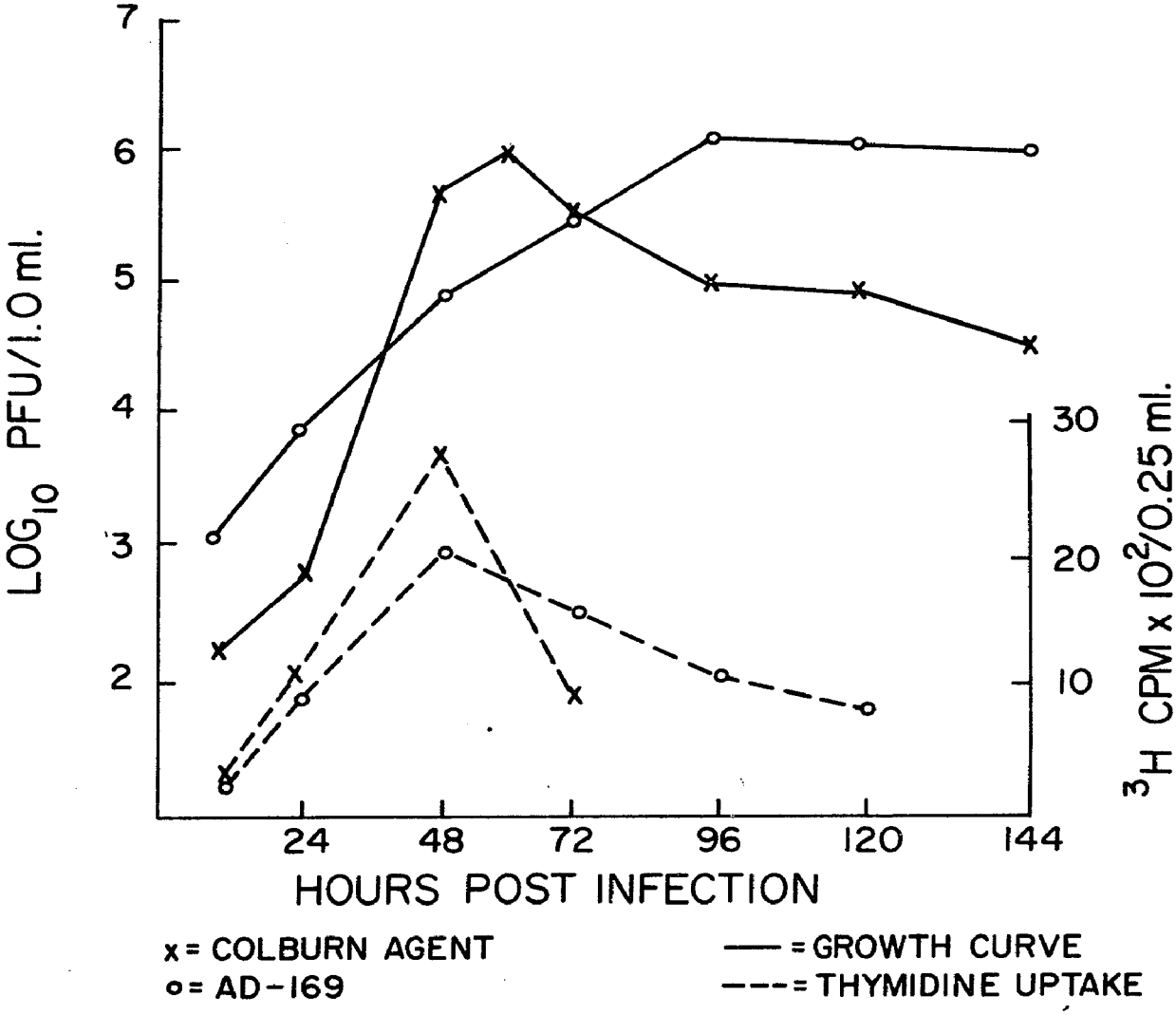
The Cowdry Type A intranuclear inclusions produced by Col virus in HF and HEK cells are presented in Figure 3. The more detailed morphology of the inclusions produced in HF cells is pictured in the upper panel. The fragmented cellular chromatin and nucleoli are seen against the nuclear membrane which is divided from the compact deeply stained inclusion by a clear halo. The lower panel displays similar inclusions produced in HEK cells infected with Col virus.

Kinetics of Replication

The Col virus adapted very slowly with total virus production being 3-3.5 log $TCID_{50}$ per 0.2 ml after 35 passages in HF cells; after the 60th passage, infectivity titers ranged from 5-6.5 log $TCID_{50}$. At this time the kinetics of Col replication could be compared to that of the adapted human CMV, AD169. The results of such comparisons are illustrated in Figure 4. Production of viral DNA following inoculation of Col and the human CMV prototype were similar although the Col infected cells appeared to incorporate thymidine more rapidly and to a slightly higher degree than did AD169 infected cells.

Figure 3. Intranuclear inclusion produced by the Col virus in human fibroblasts (Upper panel, H and E stained, 400 x) and human embryonic kidney cells (Lower panel, H and E stain, 100 x).

Figure 4. Comparative kinetics of growth and thymidine uptake for the Col agent and AD169 isolate of human CMV in human fibroblasts. Virus quantitation represents extracellular virus only.


7
6
5
4
3
2
LOG10 PFU/1.0 ml.
30
20
10
3H CPM x 10²/0.25 ml.
24
48
72
96
120
144
HOURS POST INFECTION
x = COLBURN AGENT
o = AD-169
—— = GROWTH CURVE
--- = THYMIDINE UPTAKE

With both agents, thymidine uptake was first detected at 24 hours, peaked at 48 hours and continued at reducing rates thereafter. The fall-off rate for Col exceeded that of AD169 and was associated with a more rapid advancement of the CPE. As shown in this same figure, virus production mirrored the thymidine uptake patterns, at least in the early stages of viral replication and indicate a similarity in growth between Col and human CMV when grown in human fibroblasts. Virus eclipsed between 6-12 hours after adsorption and first reappeared at 24 hours. Levels of Col extracellular virus peaked at 48 hours while AD169 did not do so until the fourth day.

Animal Pathogenicity

None of the mice injected with Col or the human CMV agents died or showed any signs of disease during the 30 day observation period. Virus could not be recovered from the heart, spleen, brain, lung or kidney of animals sacrificed on days 10, 20 or 30 post-inoculation.

Biologic Characterization and Comparative Studies

The results obtained thus far had indicated Col virus to be a herpesvirus, most closely related to CMV. Its ability to grow in tissues of human, simian and rabbit origin violated the species specificity criteria commonly ascribed to human CMV. The only non-human CMVs with characteristics similar to Col virus were those of simian origin; thus, the biologic characteristics of Col virus regarding cytopathic and growth potential were examined more critically. Comparisons to simian CMV strains from African green and Rhesus mon-

keys were included since these represent agents most likely to be encountered as a result of laboratory contamination. Two human CMV isolates, which apparently represent widely separated strains were also compared to Col virus.

Studies in Human Fibroblasts

As seen in Table 4, all agents grew well in HF cells as evidenced by the amounts of extracellular virus released from infected cells. The titers expressed are those routinely attained in our laboratory as the passage levels indicated. The simian isolate, CSG, grew better than did Col or the human agents, while the other simian agent, 68-1, appeared to replicate less efficiently as indicated by the lower quantities of infectious virus released and the prolonged incubation period required to produce maximal CPE.

Since all agents replicated well in this tissue, growth curves for extracellular virus were constructed for AD169, CSG and Col virus and are illustrated in Figure 5. All three agents displayed an eclipse period of 24 hours after which time Col and CSG replicated more rapidly than did AD169. The greatest amount of extracellular virus was present at 96 hours for Col and CSG, whereas AD169 required 144 hours to reach peak titers. A decrease in titer of infectious virus after 96 hours occurred with both Col and CSG, while levels of AD169 remained relatively stable during the 24-hour period after maximal titers were reached. Although CSG produced CPE somewhat more rapidly than Col, the growth kinetics of these agents were virtually synchronous.

The foci (plaques) produced early in the course of infec-

Table 4

Average Production of Extracellular Virus and Maximal Cytopathic Effect Following Adaptation of Viruses to Growth in HF Cells

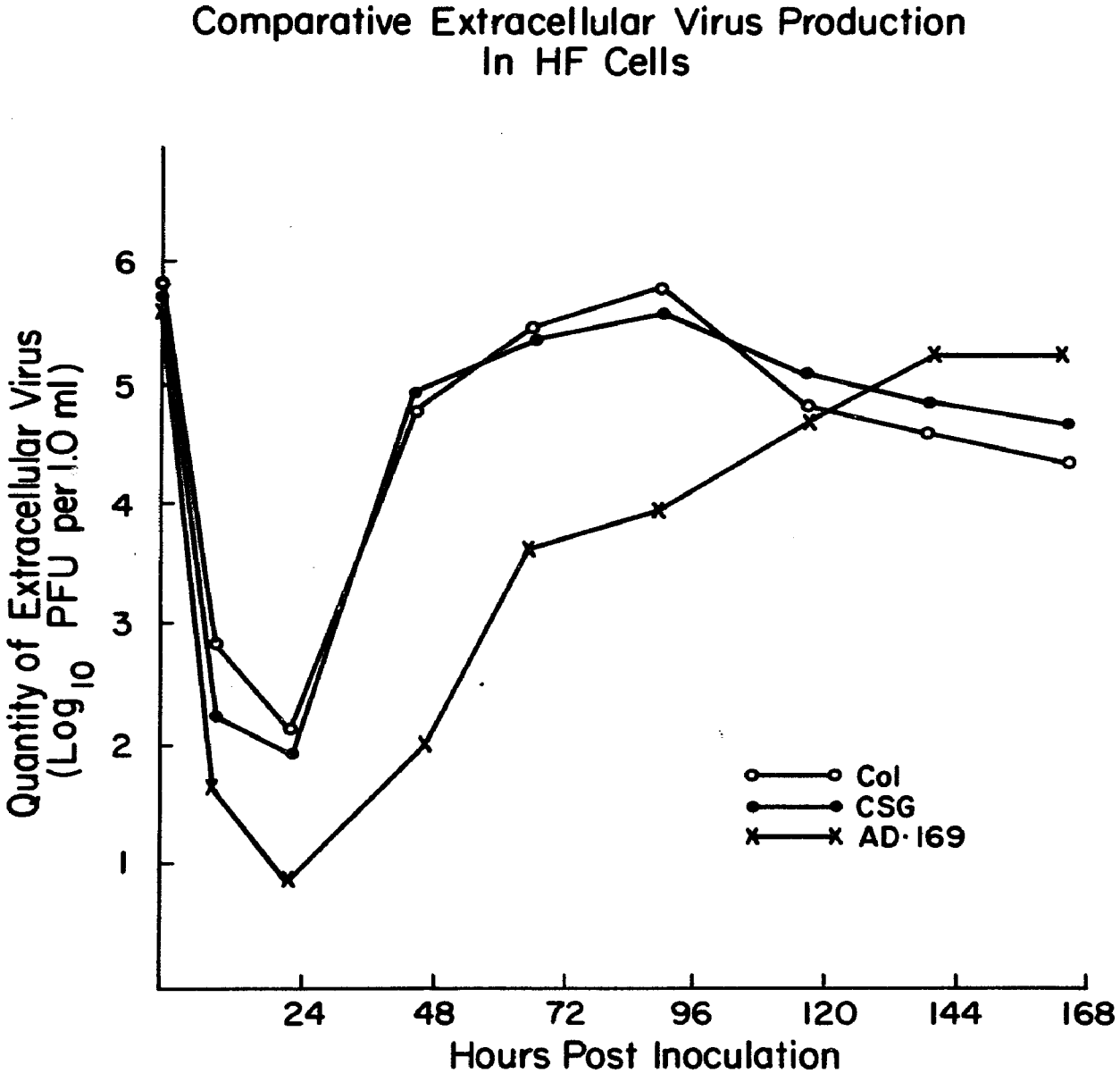
Virus	Source	Number of Passages in HF	Quantity of Extracellular Virus (1)	Average No. of Days*
Colburn	Human	75	6.0-6.5 #	4
CSG	AGM	325	6.5-7.0	3
AD169	Human	200	5.5-6.0	7
Davis	Human	150	5.5-6.0	7
68-1	Rhesus	18	4.5-5.0	14

(1) At time of maximal output (CPE 80-90%).

*Days required for CPE to involve 80-90% of cell monolayer.

#Titer expressed as Log_{10} PFU/0.2 ml in supernatant fluids. Range derived from quantitation of infectivity at 3 different passage levels.

Figure 5. Growth curves of Col, AD169 and CSG in human fibroblasts.

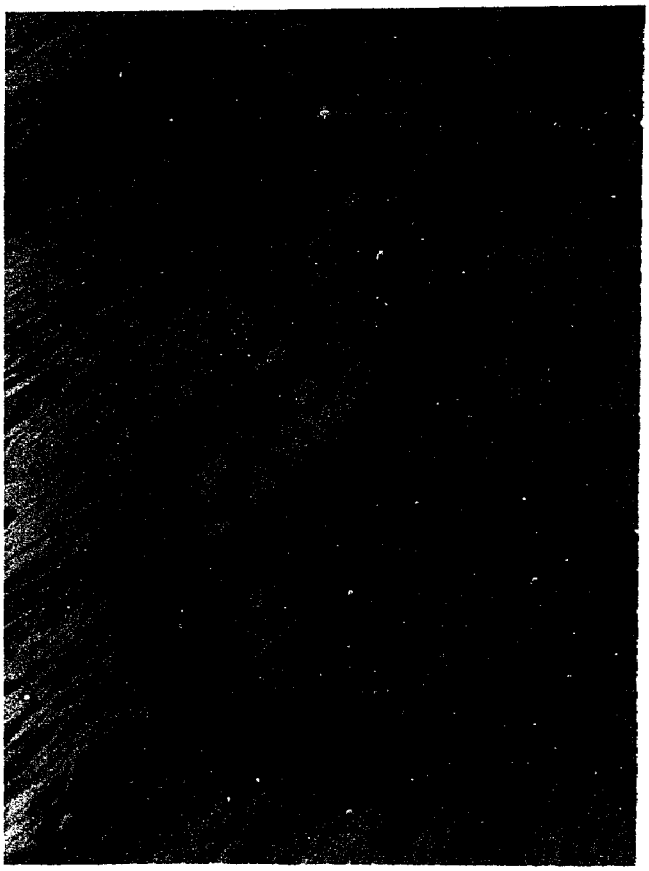

Comparative Extracellular Virus Production
In HF Cells
Quantity of Extracellular Virus
(Log10 PFU per 1.0 ml)
6
5
4
3
2
1
24
48
72
96
120
144
168
Hours Post Inoculation
Col
CSG
AD·169

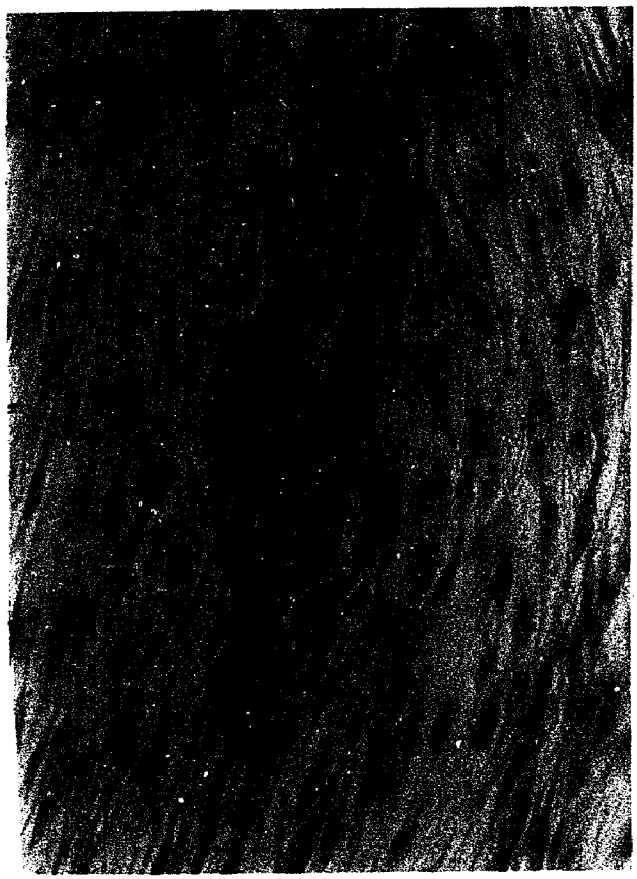
tion of HF cells are depicted in Figure 6. In roller tube cultures, the early CPE of all agents, with the exception of 68-1, was focal in nature. AD169 CPE remained focal for a longer interval than those produced by the other agents. Foci of AD169 were composed of swollen, refractile cells which retained, in the main, the fusiform morphology of the surrounding fibroblastic cells. The plaque morphology of AD169 infected cells is seen in the upper right quadrant of Figure 6. The majority of the cells remained in close contact with adjacent cells rather than separating and maintained their original orientation within the monolayer. Foci thus appear as deeply stained, solid fusiform plaques. The other human strain, Davis, produced foci characterized by more marked rounding of infected cells with some involved cells becoming perpendicular to the grain of the monolayer as seen in the lower right portion of the figure. Plaques, though solid and deeply stained, were more rounded in appearance due to the failure of infected cells to follow the contour of the cell sheet.

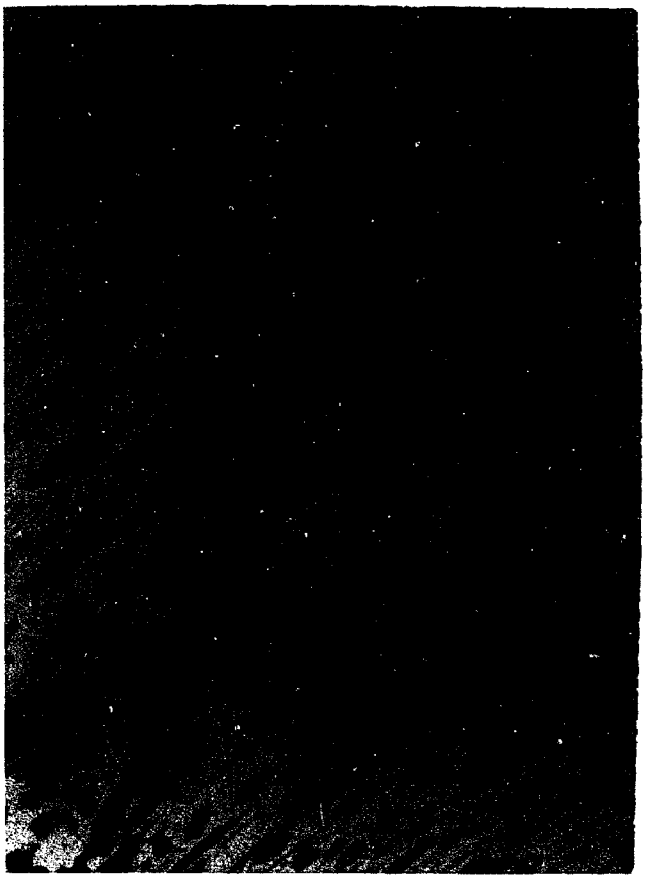
Following infection by Col, cells again became rounded like Davis but apparently lysed more quickly than those with involvement by either human CMV. Thus, central, punched-out areas, which contained scattered cellular debris as the foci spread, characterized early Col plaque formation. Col plaque morphology is presented in the upper left portion of the figure and contrasted sharply to the solid appearing state of human CMV foci, particularly early in the course of infection.

CSG infection caused focus formation earlier, and the CPE

Figure 6. The plaque morphology of Col, Human and Simian CMVs in human fibroblasts. Human CMV isolates AD169 (upper right) and Davis (lower right). Col virus (upper left) and simian CMV isolate, CSG (lower left). H and E stained, 100 x.







became generalized more rapidly than with the human strains. The involved cells again rounded, but the older, central cells of the foci did not lyse; instead they adhered to one another forming chains of clumps rather than amorphous debris. A representative plaque formed by CSG is seen in the lower left quadrant, and these plaques were usually larger than those produced by the other viruses at the same time interval after infection.

The development of the polar body also differed for Col and CSG infection. With the former, this structure was more compact and prominent when the intranuclear inclusion was mature and remained in close proximity to the kidney-bean shaped nucleus. Polar body (intracytoplasmic inclusion) development was more prominent during earlier phases of infection with CSG when the nuclear inclusion had not yet coalesced. Upon maturation of the nuclear inclusion, components that represented the polar body were more generally dispersed throughout the cytoplasm and thus were much less distinct and the nuclei were not as often beanshaped.

The CPE associated with 68-1 was distinguishable from the others because of a tendency to cause generalized rather than focal cell involvement and its plaque morphology was therefore not compared.

Studies in Rabbit Kidney

Neither human strains nor 68-1 caused productive infection of RK cells and there was no evidence of abortive infection with the 68-1. Following inoculation of RK with Davis or AD169, cellular rounding was seen in the fibroblastic components of the cell sheets reaching maximum involvement over the first 4 days without progres-

sion afterwards. The H and E stained cells had no classical herpetic inclusions. The chromatin was somewhat clumped though it remained dispersed and a large number of the cells were undergoing mitosis as seen in Figure 7. The same increase in mitotic index was noted with RK cells inoculated with the Davis strain; thus, these agents may have stimulated the RK cells to undergo mitosis rather than producing specific CPE. Infection could not be established upon transfer of these cells back into HF cells.

In contrast, both Col and CSG caused productive infection of RK cells. Their capacity to replicate extracellular virus is compared in the growth curves presented in the upper part of Figure 8. After a 24-hour eclipse period, infectivity increased in parallel over a 6-day period to peak levels. Col infectivity exceeded that of CSG throughout this interval but only by 0.5-1.0 $\log_{10}$ PFU per 1.0 ml. On serial passage, as noted in the bottom of this figure, Col also maintained somewhat greater viral output on 10 serial passages, likely as a reflection of its increased ability to generate extracellular virus seen in the growth curve.

The cytopathology of Col and CSG viruses produced in RK is shown in Figure 9. The cell pathology of these agents were similar and like their CPE in other systems. The cells were enlarged and multinucleated probably as a result of fusion. The nuclei contained Type A Cowdry intranuclear inclusions in various stages, mostly mature. One gross difference was notable quantitatively; both viruses caused vacuolization of the cytoplasm of some involved cells but Col did so with much greater facility.

Figure 7. Appearance of rabbit kidney cells following inoculation with AD169. Upper panel: Rounded cells within the fibroblastic components of the rabbit kidney tissue. H and E stained 100 x. Lower panel: Higher magnification of the same rounded cells seen in the upper panel. These cells appear to be in mitosis rather than a result of typical CMV induced CPE. H and E stained, 400 x.

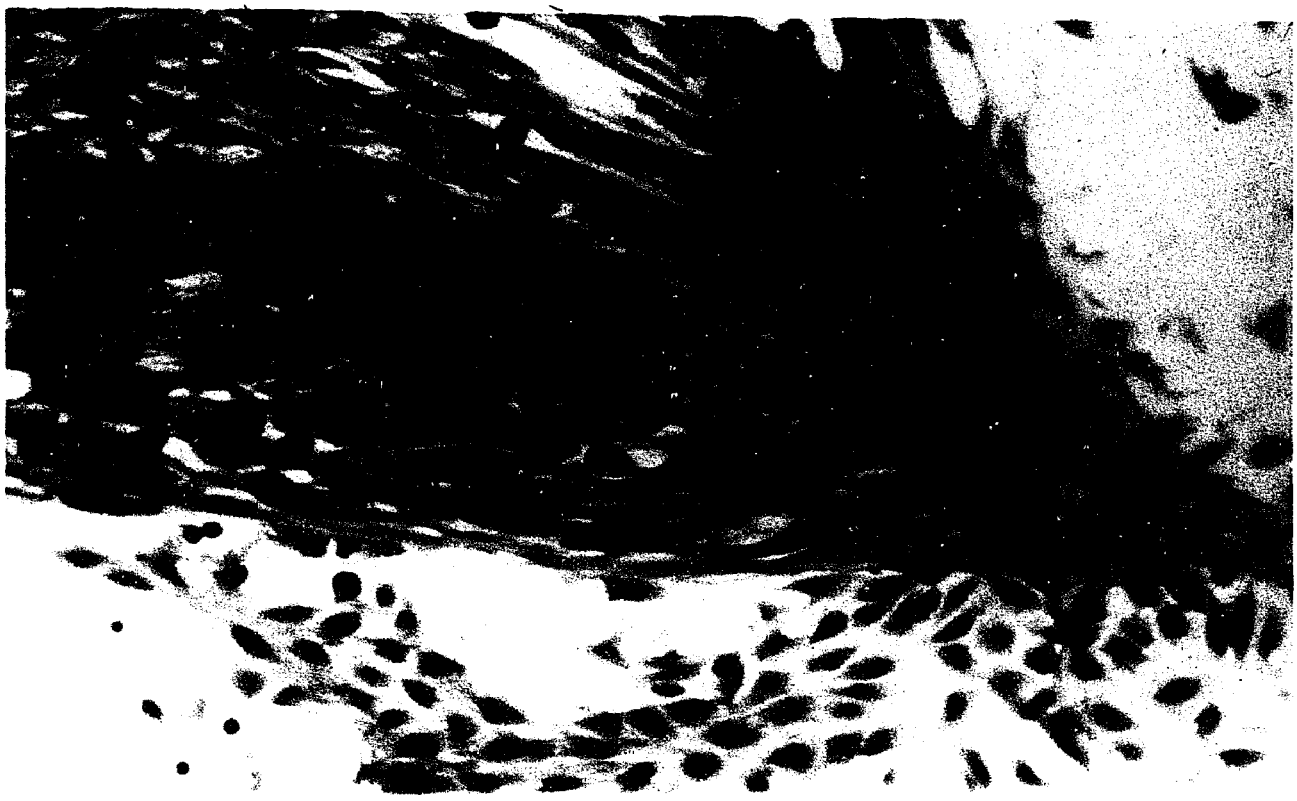


Figure 8. Amounts of infectious extracellular virus produced by Col and CSG strain of simian CMV in rabbit kidney cells. Upper panel: Growth curves. Lower panel: Amounts of infectious virus measured during serial passages, 1, 5 and 10.

Amount of Infectious Extracellular Virus Produced in Rabbit Kidney Cells

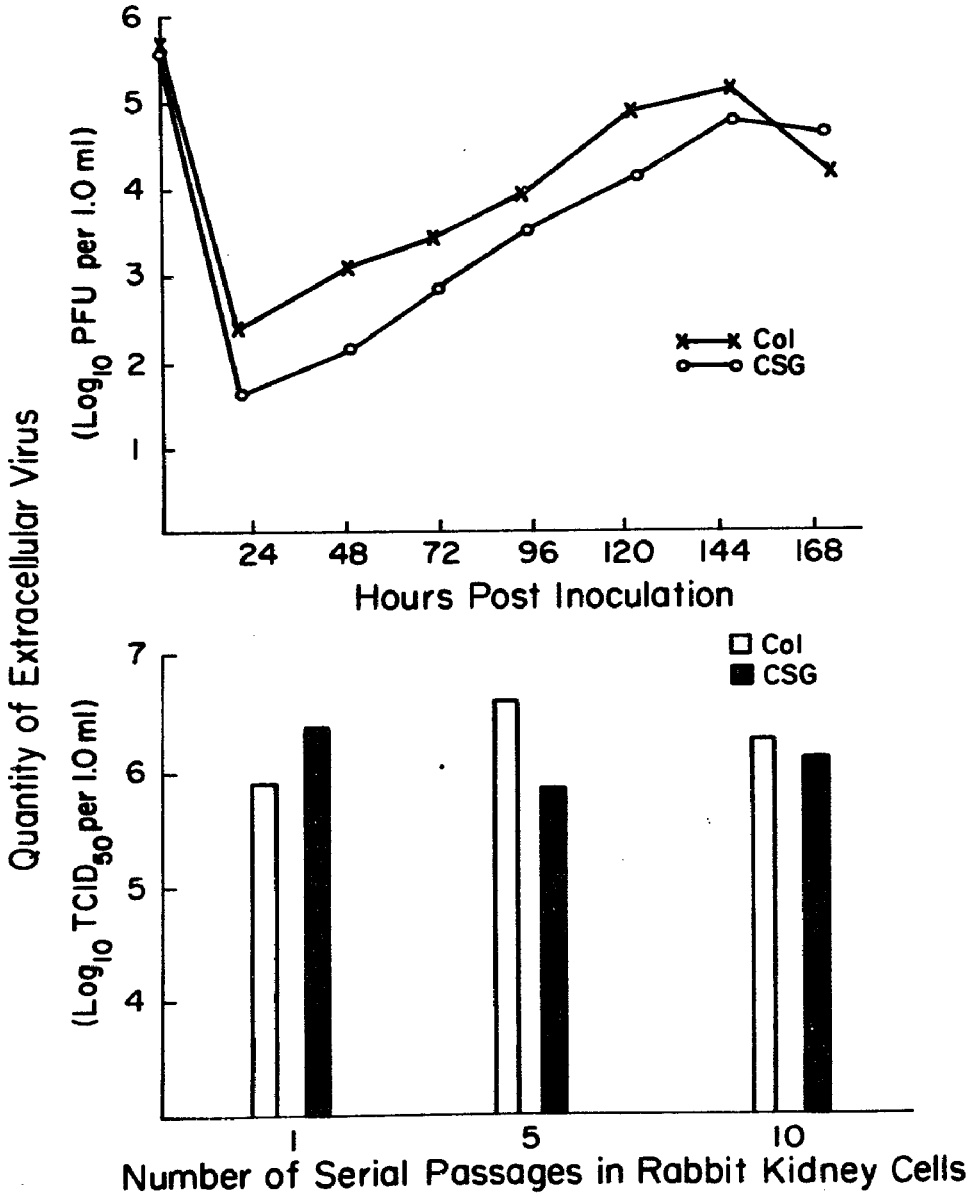


Figure 9. Cytopathology of Col virus and the CSG strain of simian CMV in rabbit kidney cells. Upper panel: Col virus induced cytopathology with typical vacuoles and cell fusion. Lower panel: CSG induced cytopathology with formation of multinucleated giant cells. Both agents produced Type A intranuclear inclusions and CPE which was very similar with the exception of more vacuolization in the Col virus infected cells. H and E stained, 540 x.

Comparative Studies in BSC-1

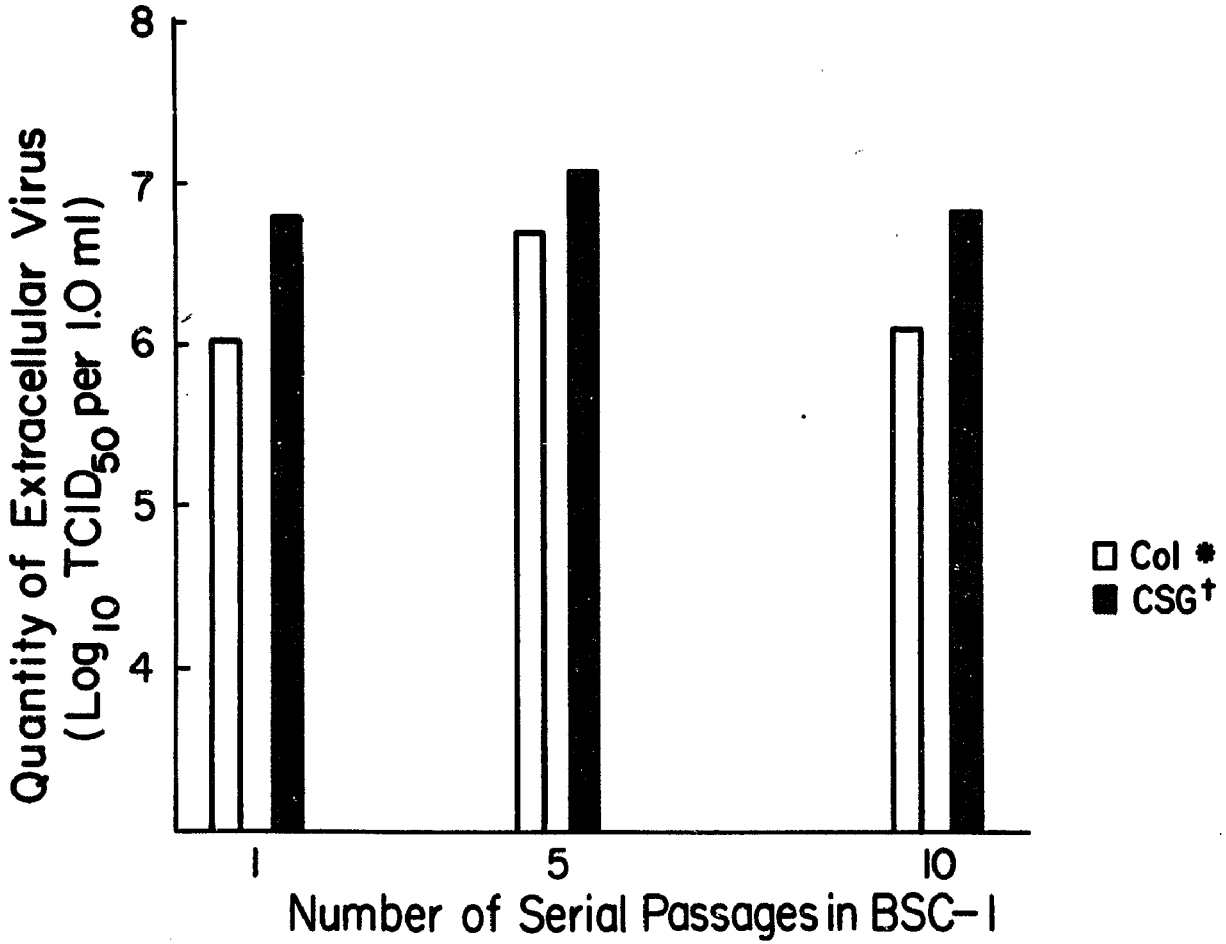
The Rhesus and the two human strains failed to infect either productively or abortively nor did they stimulate mitosis in BSC-1 cells. In contrast, both Col and the CSG strain productively infected these cells but in a very dissimilar manner. CSG infection was easily established by inoculation of extracellular virus, even with low MOI, and infection could be serially maintained in this way as noted in Figure 10. High levels, approximately 10^7 $TCID_{50}$ per 1.0 ml, of CSG extracellular virus were generated under these conditions. With Col, however, serial infection could not be maintained with inoculation of extracellular virus, except through 3-4 serial passages beginning with an MOI of 1. Decreasing levels of virus were generated on serial passage until none was detectable by the fifth passage. For this reason, one-step growth curves could not be compared.

Productive Col infection of BSC-1 cells could be maintained serially if intracellular virus (infected cells and supernate) was transferred. Under these conditions 10^6 $TCID_{50}$ per 1.0 ml of extracellular virus was synthesized at maximal output. Even when conditions were maximized, Col virus production was less than that of CSG using extracellular virus input only.

The CPE produced by Col and CSG in BSC-1, as seen in Figure 11, was again similar and resembled the CPE seen in RK cells. Unlike HF, foci formation was much less distinct in BSC-1 and RK cells, so plaque morphology was not compared in these systems.

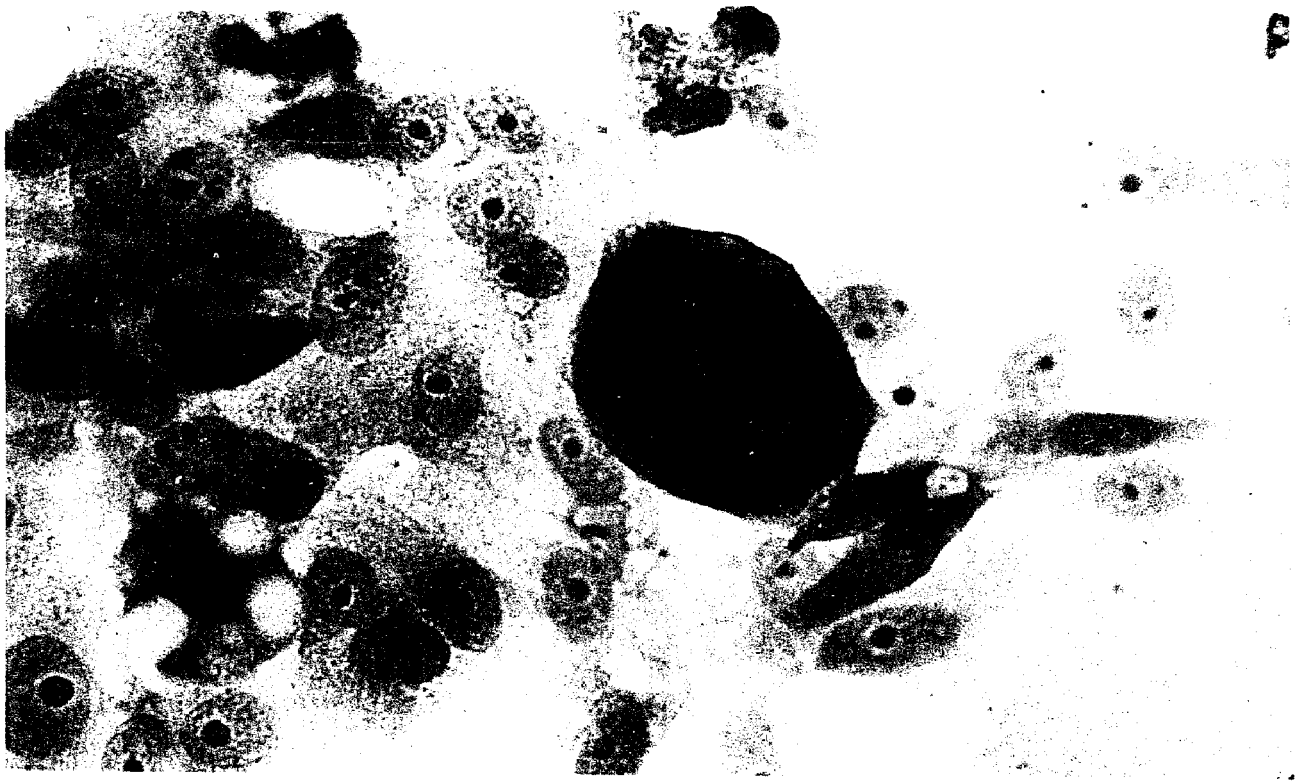
Figure 10. Amounts of infectious extracellular virus produced in BSC-1 cells by Col and the CSG strain of simian CMV. Virus measured at serial passages 1,5 and 10 under the passage conditions indicated.

Amount of Infectious Extracellular Virus Produced in BSC-1



*Cells and Supernatant Fluid Used for Serial Passage
†Clarified Supernatant Fluid Used for Serial Passage

Figure 11. Cytopathology of Col virus and the CSG strain of simian CMV in BSC-1 cells. Upper panel: CSG virus induced CPE. Lower Panel: Col induced CPE. Both agents produce intranuclear inclusions and giant cell formation with no clear differences between their cytopathology. H and E stained, 540 x.



Serologic Characterization and Comparative Studies

The results of biologic studies had indicated Col virus to be more closely related to the simian CMV of African green monkeys based on host cell range although it had been possible to separate these agents in both HF and BSC-1 cell cultures.

The serologic properties of Col virus were therefore examined to determine the antigenic relatedness between Col virus and prototype human and simian CMVs. The fluorescent antibody (FA) and neutralization (NT) tests were chosen since both had been shown to be specific for CMV. The NT test was necessary since the hyperimmune animal sera available could only be utilized in this manner and the FA test was used because of its increased sensitivity and ease of application to screening multiple human sera.

Cross Neutralization Using Hyperimmune Sera (Table 5)

When employing the 60 percent plaque reduction end point, as advocated by Benyesh-Melnick, hyperimmune sera prepared in goats and primates against human AD-169 were specific for homologous virus and the human Davis strain at equivalent levels. However, when actual percent neutralization was examined, these antisera cross-reacted better with Colburn than with either simian CMV, but at low levels. The rabbit hyperimmune sera prepared against AD-169 and Davis showed a broader range of cross-reactivity. Rabbit anti-Davis reduced plaque formation of Col by over 60 percent at a dilution 1/32 whereas that prepared against AD-169 reduced the plaques produced by Col and CSG over 40% at a 1/16 final dilution. When total results were reviewed, Col appeared more closely related antigenically to

Table 5

Comparative Cross Neutralization of Col, Human, and Simian CMV Using Hyperimmune Sera

Antisera	Antigens				
	Col	AD-169	Davis	CSG	68-1
Goat anti-AD-169	<4	512*	512	<4	<8
Primate anti-AD-169	(8)	256		<4	<8
Rabbit anti-Davis	32	512	1024	<4	
Rabbit anti-AD-169	(8)	512	512	(8)	<8
Goat anti-2757	8	<8	<8	128	<8
Primate anti-2757	<8	<8		512	<8
Rabbit anti-Col (1)	512	(8)		32	
Rabbit anti-Col (2)	512	(8)	<8	32	<8
Renal Transplant Sera					
#1	32	1024		<8	
#2	64	256		<8	

*Reciprocal of neutralization titer (60% end point)

(8) Greater than 40% neutralizing end point at final serum dilution of 1/16

(1) prepared in Boston

(2) prepared at UAB

human CMV than did the simian viruses but the relationship was quite distant in either case.

The goat antisera prepared against African Green Monkey CMV (GR-2757) cross-reacted with Col at a low level, and only minimally, if at all, with human CMV, while the primate counterpart was specific. Thus, Col appeared to be very distantly related to this AGM virus, just as it was to human CMV.

Antisera prepared against Col in our laboratory and by Dr. Waner in Boston cross-reacted with both simian and human CMV with the former showing a slightly greater degree of antigenic homology.

Serum collected from a renal transplant recipient actively infected by human CMV neutralized Col but at levels 4 to 32-fold less than it neutralized AD-169 and failed to react with simian virus at the lowest dilutions employed. Thus, human antibody recognized Col better than simian virus, although it is unclear whether this antibody is Col specific or cross-reacting antibody to human CMV.

Serologic Comparisons Using Col Sera (Table 6)

Four weeks after onset of symptoms (at time of brain biopsy), a high level of FA antibody against Col virus was detectable in the patient's sera. It rose 4-fold over the ensuing 23 weeks and stabilized. A similar response was seen against the AD-169 strain, though levels were 4-fold less than those against Col virus at each sample period. Reactivities against the other 6 human strains were also present but at levels 2 to 32-fold less than those against Col. They remained unchanged throughout the sampling period, while only low level reactivity was observed with simian CMV and none against V-Z.

Table 6

Fluorescent Antibody Tests Comparing Col,
Seven Human CMV Strains, Simian CMV and Varicella-Zoster Virus

Colburn Sera	Col	AD-169	Davis	1+	2	3	4	5	CSG(2)	V-Z
#1 4 weeks (1)	512*	128	256	64	64	256	256	64	8	<8
#2 25 weeks	2048	512	256	64	256	256	256	64	8	<8
#3 27 weeks	2048	512	256	256		256	256	64	8	<8

*Reciprocal of highest dilution reactive in IFA test

(1) Time post onset of symptoms when sera obtained

(2) African green monkey CMV

+Numbered strains refer to recent clinical isolates of human CMV

Complement-fixing antibody levels employing both Col and AD-169 antigens increased from 8 to 8 over the study interval; other antigens were not compared by this method. As summarized in the upper panel of Table 7, neutralizing antibody was not detectable against homologous virus, AD-169 or CSG in Col sera at a 1:4 dilution in spite of the reactivity detectable in the other serologic test procedures.

Serologic Comparison Using Human Sera Collected After An Apparent Laboratory Acquired Infection by Col Virus (Table 7, Panel 2)

One individual (FL), who was seronegative for CMV antibody, apparently became infected by Col virus during laboratory investigations. This conclusion was based on the obvious FA seroconversion specifically to Col virus. In spite of the high levels of Col antibody, no cross-reacting FA antibody was detectable against either human or simian CMV. In addition, neutralizing antibody has not developed against any of the CMV, including Col, throughout the study interval which has spanned almost 3 years. This peculiar lack of neutralizing antibody response was also noted in the biopsied patient's sera.

Serum collected from another laboratory worker, B.B. (Table 7), reacted equivalently in the FA test with Col and AD-169 and at a much lesser level against simian, like the Col sera. In this case neutralizing antibody against human CMV, but not Col or simian virus was demonstrable. Relatively low but specific Col FA antibody without a neutralizing counterpart was also detected in the sera collected from a child with no known exposure to Col virus (A.S., Table 7). In 60 serum samples lacking antibody to human CMV, no reactivity was

Table 7

Comparative Serologic Testing Using Fluorescent and Neutralization Methods and Various Human Sera

Sera	Antigens						
	IFA				Neutralization		
	Col	AD-169	Davis	CSG	Col	AD-169	CSG
Colburn							
1	512*	128	256	<8			
2	2048	512	256	8	<4	<4	<4
3	2048	512	256	8	<4	<4	<4
Laboratory Acquired Infection (Col virus)							
F.L.							
3/20/73	<8	<8	<8	<8			
10/26/73	1024	<8	<8	<8	<4	<4	<4
2/26/74	1024	<8	<8	<8	<4	<4	<4
1/20/75	256	<8	<8	<8			
Other Persons							
A.S.	128	<8	<8	<8	<4	<4	<4
B.B.	256	256	256	8	<4	64	<4

*Reciprocal of highest dilution sera with reactivity

detectable against either Col virus or CSG.

Serologic Relatedness Detectable By FA Testing

To better understand the broad serologic cross-reactivity of FA antibody seen in the Col sera, similar comparative studies using human sera collected from adults and infants with proven CMV infections were performed. The results are summarized in Table 8. Sera were collected from patients with maternally transferred, early and late naturally produced antibodies. Some were actively excreting CMV (infants with congenitally and natally acquired infections), while others excreted virus intermittently or had ceased excretion (adult female with cervical excretions). Data concerning the patient population from whom these sera were obtained have been previously detailed (Reynolds et al., 1973).

As noted in Table 8, with human CMV infections and regardless of the type, FA antibodies cross reacted with the two prototype human strains AD-169 and Davis, with differences no greater than 4-fold. Antibody levels against Col virus, however, were always much lower, 4 to <1000 (average 34) fold, and, if present, even less against the simian CMV, 16 to <2000 (average 217) fold. Comparative studies with pooled human sera collected from infants chronically infected by CMV reflected the same pattern of presumed cross-reactivity for FA antibody. Neutralizing antibody, as noted with the pooled sera, was specific for human CMV.

The pattern of FA antibody (Tables 6 and 7) in the Col sera differed considerably from that accompanying human infection with classical strains of CMV, as described above. Levels of specific

Table 8

Comparative Serologic Results Using Col, Human and Simian CMV Using Sera Collected from Persons with Proven Classical CMV Infections

Sera	Antigens						
	IFA				Neutralization		
	AD-169	Davis	Col	CSG	AD-169	Col	CSG
1	16384*	4096	16	8			
2	4096	1024	64	8			
3	256	256	16	8			
4	1024	256	64	8			
5	1024	256	64	8			
6	1024	1024	64	16			
7	256	1024	64	16			
8	4096	1024	64	16			
9	1024	4096	64	16			
10	16384	4096	64	16			
11	16384	16384	64	16			
12	1024	1024	256	16			
13	256	256	16	8			
Geometric Mean Titer	1734	1258	51	8			
Pooled Human Sera #	4096	1024	256	8	128	<8	<8

*Reciprocal of highest serum dilution

#Sera collected from 5 infants chronically infected by CMV

Col FA antibody exceeded those against 7 human strains, including AD169 and Davis, without a concomitant increased reactivity against simian CMV. The Col sera failed to neutralize homologous virus, human or simian CMV in spite of the presence of relatively high quantities of FA antibody, again unlike sera collected from patients with classical CMV infection.

Serologic Comparisons Using Sera Collected From Renal Transplant Patients

The results obtained in FA tests with sera from renal transplant patients are summarized in Table 9. Normal hosts of varying ages but with common forms of CMV infection had high levels of FA antibody against AD169. The geometric mean titer was 1734, and titers ranged from 256-16384. All these sera were positive when tested against Col antigens but always to a lesser degree, the geometric mean titer greater than 4 two-fold dilutions less. Only 60 percent of these same sera were positive against CSG antigens and to a lesser degree than that seen to Col by a factor of approximately 3 dilutions.

In the renal transplant group the geometric mean titer against human CMV was essentially equal considering the 2-fold variability routinely allowed in serologic determinations, as was the titer against CSG. In contrast, titers against Col virus increased toward equivalency with those against AD169 until only 2 dilutions separated the titers. In the transplant patients, the mean titer of antibody against Col antigens was more than 3 dilutions greater than similar levels in the normal hosts. All of this suggests the possibility of either reactivation or exaggeration of a pre-existing Col

Table 9

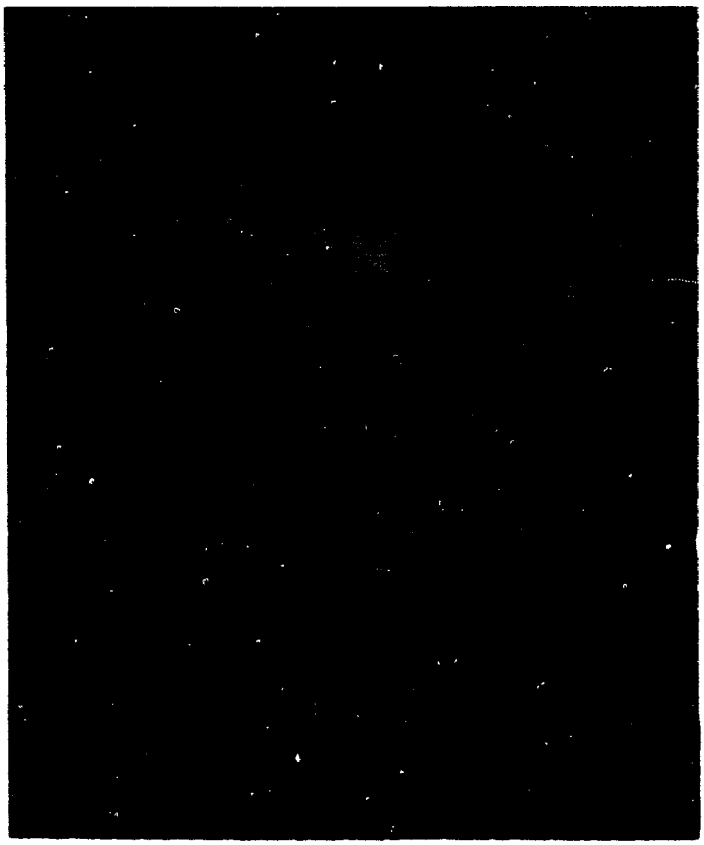
Comparative Results of Fluorescent Antibody Response to Col, Human and Simian CMV in Normal and Immunosuppressed Patients

Sera		Antigen		
		AD169	Col	CSG
Silent Infections of Normal Patients N = 13	Number Positive	13	13	8
	Geometric Mean Titer	1734	51	8
	Range	256-16384	16-256	8-16
Infections Immunosuppressed Renal Transplant Patients N = 7	Number Positive	7	7	3
	Geometric Mean Titer	1024	256	12
	Range	256-16384	64-4096	8-16

infection in renal transplant patients.

One final result of FA tests using human sera against antigen preparations of Col virus, human and simian CMV is shown in Figure 12. Presented in this figure are representative patterns of fluorescence observed for AD169, Col and CSG when the same sera was simultaneously employed. The photomicrograph at the top shows the "whole cell" pattern of staining seen against AD169. Using this relatively high serum concentration, the entire surface of infected cells was stained, although it was possible to discern restricted compact areas within the cell, probably synonomous to the inclusions seen with H and E staining. In the center the staining of Col virus infected cells appeared more intracellular, with an intensely stained bean-shaped inclusion occupying the upper portion of the cell and a prominent polar body visible below the concave side of the inclusion. The more prominent appearance of the polar body was previously mentioned in describing results of H and E stained cells as being much more common in Col virus infected cells and the FA studies support this observation. The lower picture is a CSG infected cell which, like Col, displayed more intracellular staining with the polar body being more diffuse, when present. These results utilized sera from a renal transplant patient; however, identical patterns were observed with all human sera which had neutralizing antibody to CMV.

Figure 12. Patterns of fluorescent staining of acetone fixed HF cells infected with human CMV, AD169 (top), Col (middle) and simian CMV, CSG (bottom). Antibodies in this human serum bind to both the membrane and intracellular structures of AD169-infected cells. Antibody binding is restricted to intracellular structures, primarily intranuclear inclusions in Col and CSG infected cells. The polar body (arrow) appears more spherical and condensed in the Col-infected cell whereas only diffuse fluorescence is present in this area of the CSG-infected cell.



V. DISCUSSION

A summary of the physico-chemical and biologic properties of Col virus is presented in Table 10 where they can be compared to those of other lytic herpesviruses of man, namely HSV and V-Z. The classification of Col as a member of the herpesvirus family is based on the first five tabulated characteristics which are common to all members of this family. The suppression of Col with IDU indicates the genetic material is DNA. This finding was confirmed by Dr. Clyde Goodheart who extracted the DNA and determined its buoyant density to be 1.710 g/cm^3 (personal communication), a value within the range of buoyant densities reported for other herpesviruses, as shown at the bottom of the table. Ablation of Col infectivity by ether, acidity and temperature elevation all suggest a coat composed of lipids and proteins essential to infectivity, again a property of the herpesviruses. Actually, the consistent production of Type A Cowdry intranuclear inclusions in multiple cell types provided the original clue to the taxonomy of Col virus since, to date, only herpesviruses have been reported to possess this characteristic when assessed in cell culture.

Besides its buoyant density, Col virus can be distinguished from HSV on the basis of cell pathology, growth kinetics and lack of mouse virulence. Both Col and HSV lack species specificity, a dominant characteristic of CMV strains. The host cell range of Col is,

Table 10

Physico-chemical and Biologic Comparison Between Colburn Virus and Other Lytic Herpesviruses of Man

	Col	CMV	HSV	V-Z
IDU Suppression	+	+	+	+
Ether Sensitivity	+	+	+	+
Acid Sensitivity	+	+	+	+
Thermal Lability	+	+	+	+
Cell Pathology				
Type A Cowdry intranuclear inclusions	+	+	+	+
Cytomegaly	+	+	-	-
Polar body	+	+(a)	-	-
Growth				
Replicative cycle				
rapid	-	-	+	-
slow	+	+	-	+
Extracellular virus production				
early	+(b)	-	+	-
adapted	+	+	+	±
Species Specificity in vitro (productive infection)	-(c)	+	-	-
Mouse Infectivity	-	-	+	-
Marmoset Infectivity	+	-		
DNA buoyant density (d)	1.710(e)	1.716	Type 1 1.726 Type 2 1.728	1.705

(a) more evident with Col and Davis than with AD-169
(b) much less produced than with HSV
(c) host cell range more restricted than those of HSV and V-Z
(d) values in g/cm^3 in Cesium Chloride gradients
(e) determined by Dr. Clyde Goodheart and published with his permission

however, much more narrow than that of HSV, as evidenced by its failure to infect HeLa, Hep-2, CE or HA, all of which are permissive for the growth of HSV. The major difference in Col and V-Z, in addition to the buoyant densities of their DNA, is the latter's inability to maintain extracellular virus production in any of the cell culture systems employed here and Col's inability to infect HA cells. Clearly, Col differs grossly from either HSV or V-Z, biologically, and shares no common antigens with either according to serologic testing using human sera containing high levels of antibody against HSV and V-Z (personal communication, Waner).

The only other known group of lytic herpesviruses capable of infecting man is the CMV. Thus, Col was originally presumed to be a member of this group with the understanding that its host cell range both <u>in vivo</u> and <u>in vitro</u> contradicted the dictum of strict species specificity usually ascribed to human CMV. Instead, its biologic properties more closely resembled simian CMV. This raised the possibility that Col was a simian laboratory contaminant originating in the GMK cells or that the commercial HEK cells in which Col was recovered were of simian rather than human origin. Direct evidence with respect to the source of the HEK cells could not be obtained since they were not available for chromosomal analysis. The other possibility was that Col was indeed a simian CMV which had latently infected the brain of the child from whom it was recovered. Exactly how such exposure could have occurred is obscure. The two aforementioned possibilities had to be examined in order to determine if Col virus was an unusual CMV or a new herpesvirus of man.

The fact that Col was not simply a laboratory contaminant is supported by a number of different findings. If the GMK cells in the initial isolation experiment had been the source of contamination, then an agent similar to Col should have been recovered from them initially and certainly on passage, since they were handled in such a way as to maximize recovery of CMV (Smith et al., 1969). If the HEK cells, rather than the brain inoculum had been the intrinsic source, then Col should have emerged upon passage of the uninoculated control cells, but this did not occur, as noted in the original isolation history. In addition, cells from batches of both the GMK and HEK cells used in the initial isolation experiment were employed in many other experiments run concomitantly with the original isolation and simian CMV was not recovered in any of these studies.

The re-isolation of Col also provides strong evidence that the inoculum was the source in spite of the emergence of the virus in GMK cells. Again, in the re-isolation experiments, the uninoculated control cells remained negative on passage while all inoculated cells grew virus. Moreover, in both the initial and re-isolation, the cytopathic effect first appeared in close proximity to the brain mince (Figure 2). The remoteness of antigenic similarity between Col and CSG, as noted in the serologic studies using specific hyperimmune sera, virtually excludes simian CMV as a candidate laboratory contaminant since GMK cells are the only simian materials ever employed in our laboratory. Finally and most importantly, serological evidence as discussed in detail below, indicates infection of the patient by Col virus. Thus, it can be reasonably concluded that Col virus

was harbored in the brain tissue rather than in some extraneous material. This leaves the second possibility, namely, that Col virus had latently infected the child. Since no virus was isolated from any peripheral site, circulating white blood cells or spinal fluid, primary infection can be excluded and reactivation surmised. The designation of Col as either a new herpesvirus or an unusual CMV required basic knowledge of the biologic and serologic properties of Col in comparison to CMVs of both human and simian origin and, thus, such studies were undertaken.

Biologically, the ability of Col to infect tissues of simian and rabbit origin easily distinguished it from the prototype human CMV isolates, AD169 and Davis. Even in HF cells, which are permissive for both human CMV and Col virus, differences are distinctive. Col has a faster replicative cycle, maintains higher extracellular virus levels and produces unique cytopathology with resultant differences in plaque morphology. The CPE produced by Col is more comparable to that of Davis rather than AD169 because of its ability to cause cell rounding. This result is not surprising since AD169 induced CPE is not representative of that routinely produced by isolates of CMV from human infections. All 5 isolates obtained from clinical material and used in these studies also resemble Davis much more than AD169 regarding CPE. While these differences have been noted by other investigators, this information has not been incorporated into the literature. As a consequence, in many studies, AD169 has been used as the sole prototype of human CMV whereas Davis is probably a better choice for investigations aimed at establishing

biologic criteria for separating human CMV into subgroups. Though the two human strains can be distinguished on the ground of their CPE, Col can be easily separated from both of them by growth kinetics as well as CPE.

Col also differs strikingly from the 68-1 isolate of Rhesus monkeys on many of the same grounds mentioned for human CMV. However, some of these may be due to reduced cellular adaptation of this strain. Though lack of adaptation may be a factor in these differences, the low levels of virus produced in HF cells and the failure to infect RK or BSC-1 cells appear to be innate biologic properties of the Rhesus monkey CMV since these differences remain unchanged through 15-20 serial passages in our laboratory.

In contrast to the human and Rhesus monkey CMV, the biologic differences between Col and CSG (African green monkey CMV) are more subtle. Production of extracellular virus in HF cells is identical but cytopathology is sufficiently distinct in focus and plaque formation to allow separation of the two. The sequence of polar body formation also differs, being a more prominent feature in Col infected cells, especially when the intranuclear inclusion is mature. Differences in RK cells are equally subtle but likewise reproducible on serial passage. Col produces somewhat larger quantities of extracellular virus and causes more vacuolization of the cytoplasm of infected RK cells.

The most unique biologic difference between Col and CSG is expressed upon infection of our BSC-1 cells. In this instance, unlike HF and RK cells, differences in growth capacity are quite

evident, particularly in light of the fact that both agents had been equally adapted to growth in this system, after being carried for prolonged periods in HF cells. For serial propagation in this system, Col virus must be passed intracellularly whereas this need is bypassed with CSG due to the increased capacity of this agent to generate extracellular virus. This difference can be overcome in a single cycle of growth by inoculating a high MOI of Col virus and perhaps through further passages in BSC-1 Col could be adapted to better extracellular virus production as was necessary in other permissive systems. Currently, the reduced ability to replicate in BSC-1 remains the best biologic marker to distinguish Col from African green monkey CMV which requires far less adaptation for completing its growth potential.

To summarize, though Col is sufficiently distinct biologically from the human CMVs to warrant separation into a new subgroup of human virus, this was not the case with the African green monkey CMV with which Col shared many major properties, such as growth kinetics and host cell range. This suggested that Col might represent a simian CMV which had undergone mutation as a result of prolonged residence in the brain of man. Col's ability to latently infect marmosets, whereas human CMV does not, lends credence to this concept (Nigida et al., 1974). To further examine this possibility, the antigenic relatedness between Col and the two human and simian strains were compared by serological testing.

Col virus is easily distinguished from human or the two simian CMVs studied here by cross neutralization using antisera

prepared in a variety of animals with differing viral immunogens. In fact, Col is as closely related antigenically to human as to simian strains, but distantly so in either case. As suggested by the results with the renal transplant sera, high levels of human antibody against human CMV can cross-neutralize Col virus without reducing the infectivity of simian CMV. But under these circumstances, the presence of low levels of specific Col neutralizing antibody as a result of prior infection followed by immunosuppression cannot be excluded. This is particularly so since these are the only human sera thus far encountered that will neutralize Col virus including specimens collected from the patient from whom the virus was isolated, and another with what appeared to be a laboratory acquired infection.

With comparative FA testing, human antibody against human CMVs recognizes both Col and CSG, consistently reacting to a higher level with the former. But, once again, Col appears to share only a limited number of antigens with classical human CMVs and simian shares even less. This pattern more likely represents cross-reactivity between antibody to human CMV and the other agents than that seen with sera from the renal transplants since these former sera were collected when the infants with proven CMV infection were too young to have acquired Col infection postnatally and who were not exposed to simian virus.

With human sera containing antibody against human CMV, Col FA reactivity, with the exceptions noted below, is always less than that against human strains irrespective of the type of human infec-

tion. Antibody capable of neutralizing Col is also absent, even though appreciable amounts of neutralizing antibody to human CMV is detectable. Moreover, cells infected by human CMV give cytoplasmic and nuclear fluorescence with their specific antibody while Col infected cells show predominantly nuclear and polar body fluorescence. The demonstration of a greater number of cross-reacting antigens by FA testing is not surprising as this method appears to be more sensitive for the detection of CMV antigens than other available serologic procedures, certainly more so than the neutralization test.

When the cross-serologic studies are assessed as a whole, Col emerges as a unique herpesvirus that shares minor antigenic determinants with both human and simian CMV. It is more closely related to human strains than are AGM or Rhesus CMV. From the serologic studies, Col is not antigenically as closely related to simian CMV as the biologic similarities imply. The latter plus the identity of the DNA buoyant densities between Col and simian CMV nevertheless suggests considerable genetic relatedness between the two, in spite of their antigenic diversity. Genetic relatedness among Col, human and simian CMV are currently being assessed by DNA homology studies performed by Dr. Eng-Shang Huang at the University of North Carolina. In his preliminary investigations, he reports a 10-20% homology between Col DNA and human CMV DNA. Comparative studies with simian CMV DNA are not yet completed nor has DNA from the two prototype human strains and Col been compared. Perhaps these studies will help resolve whether Col virus originated from some simian source in the past or whether it is basically a new human herpesvirus. If these

preliminary results are corroborated, then Col will have to be assumed as genetically unique since, in previous studies from the group in North Carolina, simian and human CMV DNA possessed no detectable homology within the sensitivity of the test (Huang and Pagano, 1974). The antigenic makeup and the pecularities of its biologic properties indicate that Col has unique characteristics irrespective of its origins and evidence discussed hereafter indicates it is capable of infecting man.

The role of Col virus in human infection has been difficult to define. Indeed, whether it played a role in the peculiar encephalopathy noted in the patient from whom it was recovered remains a moot point. The non-specific neuronolysis and lack of specific cellular changes or even an inflammatory response in the brain, as noted in the patient's history, are not in keeping with changes usually seen as a result of lytic herpetic viral involvements. Yet, the persistently elevated levels of IgG in the cerebral spinal fluid suggest replicating foreign antigens and none other than Col virus was ever identified. The peculiar sub-acute course of the CNS disease also differs from the more acute response seen with classical herpetic CNS infection. Moreover, classical human CMV has not been recovered from brain, to my knowledge, outside the neonatal period without the addition of severe immunosuppression, such as occurs with transplantation (Schneck, 1965; Dorfman, 1973). There was no evidence of gross immunosuppression in the biopsied patient. The unique properties of Col virus coupled with the unusual host response, as discussed later, make a modified, reactivation disease process a

distinct possibility. Reactivation of measles virus with sub-acute sclerosing panencephalitis and papovaviruses with progressive multifocal leucoencephalopathy established a precedence for such a phenomenon (Horta-Barbosa, Fucillo and Sever, 1969; Narayan et al., 1973).

The difficulties just discussed, plus the lack of neutralizing antibody in the serum specimens collected from the patient over a long time interval spanning the disease course and convalescence, continue to give concern about human brain as a source of this virus, in spite of the impressive evidence, previously reviewed, in support of this contention. With regard to the possibility of human infection by Col virus without production of neutralizing antibody, data acquired following an apparent laboratory infection in myself are highly pertinent (F.L., Table 7). During the course of these studies I seroconverted to Col virus by FA without developing detectable neutralizing antibody against human CMV or Col. In addition, no cross-reacting FA antibody developed against any of the human or simian CMV's, examined with the possible exception of low levels of antibody which cross-reacted exclusively with the intranuclear antigens of the human strains. The pattern of cellular fluorescence produced by my sera differ from that seen following infection by human CMV. It is predominantly nuclear fluorescence in contrast to the whole cell fluorescence observed after infection by human strains. Thus, Col is apparently capable of infecting humans, at least in a laboratory setting, but the resultant infection is antigenically more limited than that following primary infection by classical human CMVs. The

apparent waning of my own Col FA antibody over time lends further credence to this latter concept. The finding of a similar pattern in the serum of a child (A.S., Table 7) indicates that such an infection can occur in nature.

From this limited antibody response, Col seems likely to be poorly adapted for growth in humans which leads one to believe they are not the natural primary host for this virus. Whether or not simians are the natural hosts of this agent will require far more extensive studies than have been tried to date. The intravenous inoculation of a high dose of Col virus into marmosets also produced only a limited infection. Virus excretion was never detected although seroconversion involving both FA and neutralizing antibody did occur in susceptible recipients (Nigida et al., 1974). In the natural host one might expect far more extensive viral replication than noted in the marmoset. Obviously other simian species should be examined to determine if they are the natural harbinger of Col virus.

Unlike the two circumstances mentioned above, interpretation of serologic response in Col sera (Tables 6 and 7) were complicated not only by the absence of neutralizing antibody to Col or any CMV but also by the presence of FA antibodies which were highly reactive with Col antigens as well as with antigens of multiple human strains and simian CMV. Seroconversion of complement-fixing antibody to both Col and AD169 to equivalent levels were also detected (personal observation). The critical question is were these responses provoked by Col virus, human CMV, or both? That these responses were initiated by Col virus exclusively are indicated by the following

evidence. The FA antibody levels against Col virus exceeded those against all other human strains in all serum samples and showed a slow booster type of response spanning the period of the patient's illness. This is in sharp contrast to the FA response following infection by human CMVs where levels of antibody against human strains greatly exceed those directed against Col or simian CMV (Table 8). The pattern of fluorescence with both Col and human CMV antigens was predominantly intranuclear, as noted in my sera rather than whole cell, as observed after infection by human CMV (examples of this pattern are shown in Figure 12). Finally, but most importantly, there was no neutralizing antibody present against human CMV, a situation yet to be encountered in human infection with classical CMV when FA antibodies of this order are present. With dual infection, neutralizing antibody against human strains should be demonstrable even in the absence of Col neutralizing antibodies, as suggested by data in B.B. (Table 7). The antibodies reactive against antigens of human strains in Col sera are therefore apparently cross-reacting Col virus antibodies which for unknown reasons are present in much larger quantities than those in my own sera. Attempts to prove this by cross-absorption experiments were thwarted because of technical difficulties.

At this point, it is perhaps appropriate to speculate on the peculiar nature of the Col antibody responses. Very recently, a soluble antigen, either internal or non-structural, has been detected in cells infected by human CMV. Antisera prepared against this antigen has no neutralizing capacity, will fix complement in the presence

of antigen and reacts only with nuclear antigens by FA (Waner, Budnick and Weller, 1974). Thus, it appears that this may be the cross-reacting antigen among Col, human and simian CMV. With limited viral replication, if this were the dominant antigen produced, then neutralizing antibody would be absent and complement-fixing and intranuclear FA reactivities might well be controlled by the quantitative variability of antigen production. The latter could easily differ between individuals, especially if they are secondary rather than primary hosts. In fact, even with classical human CMV infections, viral production varies considerably between individuals.

Irrespective of the reasons for the peculiar serologic response, past infection by human CMV is unlikely in Colburn himself and primary CMV infection was excluded. Reactivation of Col virus is therefore implied since all herpetic viruses are prone to this, particularly the CMV group. The Diphtheria-Pertussis-Tetanus (DPT) immunization given prior to Col's illness could have conceivably contributed to reactivation since it has been implicated as a cause for progressive CNS disease like that seen in Colburn (Griffin, 1974). The causative mechanism(s) for this association is, however, unknown so DPT's possible role in viral reactivation remains purely conjectural, just as does the role of Col virus as a causative or contributing factor to the CNS disease.

Since Col is capable of infecting man but its primary source in nature is debatable, the frequency of Col infection in the general population becomes important from a public health standpoint. The use of large scale serologic surveys for this purpose is complicated

by the high prevalence of antibody to human CMV and the complexities posed by the possible existence of two-way cross-reacting antibodies between human CMV and Col virus. To avoid problems in interpretation, 60 sera collected from adults known to be seronegative for FA antibody to human CMV were examined for Col FA antibody, but none were found. This implies that Col infection is not exceedingly common in the general population. But negative FA results among adults does not necessarily exclude prior infection by Col, especially if such infections are limited in character and are associated with subsequent waning of the Col antibody, as appears to be the case following my own infection. Thus, excluding the existence of a latent human infection by Col becomes quite difficult, if not impossible. To try to circumvent these difficulties, serologic evidence of Col virus reactivation was sought among a small number of renal transplant recipients. These patients represent a unique population for investigations on the reactivation of latent viruses, presumably due to the reduced capacity of the transplant's immune surveillance mechanisms resulting from rather intense immunosuppression. This is especially true with regard to the herpesviruses. The very preliminary results reported here suggest that Col virus may indeed be reactivated in these patients. The geometric mean titer of Col FA antibody is substantially increased over FA antibodies to either human or simian CMV when related to those seen in CMV infected immunocompetent hosts (Table 9). This selective increase suggests a booster effect as would be expected with reactivation of latent virus. Before this interpretation is acceptable, however, larger numbers of patients

must be examined with greater control of demographic variables to assure comparability of the patient populations. Nevertheless, the results are intriguing and offer one approach to the solution of the possible medical significance of Col virus.

VI. SUMMARY

Following isolation of a unique virus recovered from human brain, studies were initiated to appropriately classify the agent. It was found to be a Group B herpesvirus according to its biological attributes and specifically most closely related to CMV by virtue of its growth characteristics and cytopathic effect on cells in culture. The agent was found to be unique among strains of human CMV because of a lack of species specificity with regard to its ability to productively infect cells of both simian and rabbit origin. Biologically, it more closely resembles simian rather than human CMV, although distinct differences between it and CMV isolates from Cercopithecus and Rhesus monkeys were demonstrated. Certain of these biologic differences were shown to be characteristic and stable enough to be used as markers to distinguish this agent, now dubbed the Colburn agent (Col), from prototype human and simian strains of CMV.

Serologically Colburn proved to be even more distinct from human or simian CMV than it did biologically. Again, though, studies with different hyperimmune sera raised in animals suggested a somewhat closer relation between Col and simian CMV than between Col and human CMV when neutralization was used to determine relatedness. This finding was reversed when human sera collected after natural CMV infection were used in investigations employing fluorescence to signify antigenic relatedness.

Studies performed following an apparent laboratory acquisition of infection showed that Colburn possessed the capacity to infect man, but the serologic response suggested a relatively abortive infection in which only FA, as opposed to neutralizing, antibodies developed. Evidence was also presented that such may also occur in the natural state; in fact, studies in renal transplant patients suggested that it may occur relatively commonly, with a resultant latent infection.

The most difficult problem encountered thus far has been proving the human origin of this virus, although evidence was garnered indicating it was present in the original brain inoculum. Be that as it may, Colburn more closely resembled simian CMV which begs the question of its real origins and its relation to human disease. If originally a simian CMV, as it appears it might be, then the dictum of species specificity will have to be thoroughly re-examined. With regard to medical significance, this is far more than just a moot point, particularly with respect to our present program of administering live vaccine grown in monkey cells known to harbor CMV.

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