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Interferon and Cytomegalovirus *in vivo* and *in vitro*.* (32220)

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Cytomegalovirus (CMV) is one of several viruses that have been associated with long-term, chronic infection in humans, and with the capacity to cross the placenta and initiate chronic infection in the fetus(1). The nature of the cytomegalovirus-host interaction which may result in such persistent virus infection in the presence of an otherwise normal host defense mechanism has never been fully defined. Children chronically infected with this virus have a normal immunological response and neutralizing antibody may be demonstrated in their sera(1). Current evidence suggests that interferon may be one determinant of host resistance to viral infections(2-4). The present study was initiated to examine the capacity of cytomegalovirus to induce the production of interferon in human cells *in vitro*, as well as to determine the sensitivity of this virus to the inhibitory activity of interferon. The investigation was then extended to elucidate the effect of endogenous interferon on the rate of excretion of cytomegalovirus in the urine of children who are chronic carriers of this virus.

Materials and methods. Sindbis virus, originally an isolate from a Malayan mosquito pool, was obtained from Dr. Philip K. Russell at the Walter Reed Arbovirus Unit and was grown and assayed in chick embryo fibroblasts (CEF). Chikungunya virus (CV), a standard reference strain, was obtained from the Walter Reed Arbovirus Unit and prepared from the brains of infected suckling mice and assayed in rat embryo fibroblasts (REF). Adenovirus type 2 was obtained from Dr. Harold S. Ginsberg. Stock pools were prepared and assayed in WI-38 or human foreskin cultures. Newcastle Disease virus (NDV), Hertz strain, was provided by Dr. Samuel Baron. Stock preparations were grown in embryonated hens' eggs and assayed by the hemadsorption plaque technique in chicken embryo fibroblasts. A calf lymph strain of vaccinia virus, obtained from Dr. Karl Habel at the National Institutes of Health was prepared and assayed in HeLa cells. Vesicular stomatitis virus (VSV), Indiana strain, was obtained from the American Type Culture Collection. Stock supplies were prepared and assayed in L cells. Herpesvirus (*Herpesvirus hominis*) was a recent isolate that has been maintained in this laboratory. Stocks were prepared and assayed in WI-38 cells. ECHO 11 virus was obtained from Dr. A. D. Heggie and was prepared and assayed in monkey kidney cells. Cytomegalovirus, strain AD 169, was obtained from Dr. Wallace P. Rowe, National Institutes of Health. Stock virus pools were prepared and assayed in WI-38 cells and contained approximately $10^{3.5}$ tissue culture infective doses ($TCID_{50}$) per ml.

Stock human interferon was prepared by infecting WI-38 cultures with 5×10^6 plaque forming units (pfu) of CV. Supernatant fluids were harvested at 24 hours and processed as previously described(5). Materials to be assayed for cytomegalovirus interferon were harvested from CMV-infected cultures of WI-38 or human foreskin cells (HFS) and similarly processed. Interferon preparations were assayed by the plaque reduction tech-

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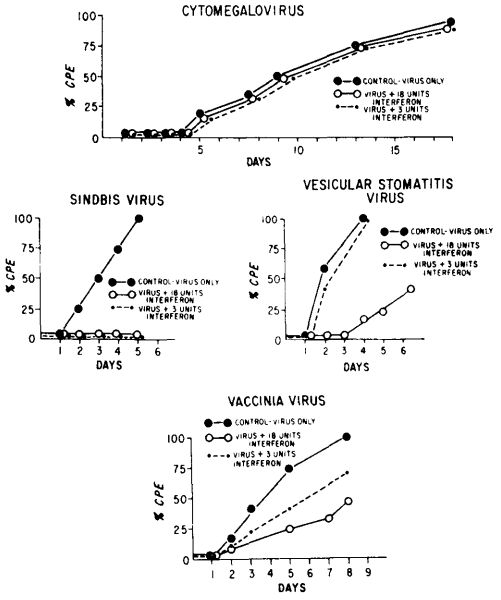

FIG. 1. Course of the cytopathic effect (CPE) of 4 viruses, plotted as estimated percent of the cell monolayer showing CPE in control cultures (●—●) and in cultures containing 3 (● - - ●) or 18 (○ — ○) units of human interferon.

nique in WI-38 or HFS cultures utilizing Sindbis or VSV as the challenge virus. Interferon preparations were characterized by: (a) stability at pH 2 for 48 hours at 4°C; (b) inactivation at 70°C for 30 minutes; (c) failure to sediment at 90,000 *g* for 2 hours; (d) trypsin sensitivity; (e) no direct inhibitory activity on the challenge virus; and, (f) species specificity.

WI-38 cells were obtained from Flow Laboratories in Rockville, Md. The human foreskin cells are a continuous line of human fibroblasts which have been maintained in this laboratory.

Results. In vitro *sensitivity.* The sensitivity of CMV to the inhibitory activity of human interferon was determined by treating cultures of WI-38 cells with a standard human interferon preparation for 24 hours prior to infection with CMV. In the first series of experiments, all cultures were maintained in control media or media containing either 18 or 3 units/ml of interferon for the duration of the experiment. Similarly treated groups of WI-38 tubes were infected with a wide range of other viruses selected to represent a spectrum of sensitivity to interferon. Limitations of available assay techniques permitted only partial control of inoculum size. Between 10^2-10^3 pfu of Sindbis, herpes and vesicular stomatitis virus, 5×10^1-10^2 pfu of vaccinia virus, and approximately 10^2 $TCID_{50}$ of CMV and adenovirus were used to challenge interferon cultures.

The results of one representative experiment are summarized in Fig. 1. All the viruses studied, with the exception of CMV, exhibited some degree of sensitivity to interferon. Sindbis virus was completely inhibited by both levels of interferon. VSV, an interferon-sensitive virus frequently used as a challenge virus in interferon assays, was sensitive to 18 units/ml, but less so to the lower concentration of 3 units/ml under the conditions of these experiments. Somewhat surprisingly, vaccinia virus was also found to be moderately resistant to interferon.

To evaluate further the degree of resistance to the antiviral action of interferon manifested by CMV, a second series of experiments was carried out utilizing a more potent interferon preparation. In these studies, 30 and 120 units/ml of interferon were added to the test groups in human foreskin cultures 24-48 hours prior to virus challenge. *Herpesvirus hominis* was included in these experiments since it has been reported to be relatively resistant to interferon (6,7) and is classified with CMV in the herpesvirus group. The data from one of these experiments are illustrated in Fig. 2. CMV again was relatively resistant to interferon although at the highest concentration of 120 units/ml the progression of the cytopathic effect (CPE) was slightly retarded. The levels of interferon used in these experiments completely protected replicate cultures of foreskin tissue against VSV and Sindbis virus with no breakthrough appearing after 2 weeks. Adenovirus which has also been considered to be relatively resistant to interferon(8), also manifested some degree of resistance with slow progression of CPE occurring in the presence of these levels of interferon. *Herpesvirus hominis* demonstrated a moderate degree of resistance to the inhibitory activity of interferon. However, progression of CPE was significantly inhibited

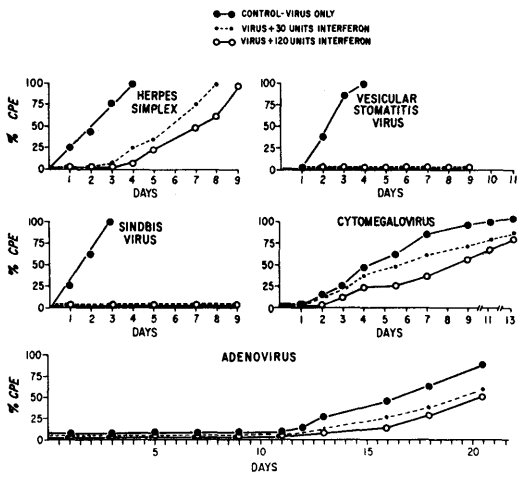

FIG. 2. Course of the cytopathic effect (CPE) of five viruses, plotted as percent CPE, in control cultures (● — ●) and in cultures containing 30 (● - - ●) or 120 (○ — ○) units of human interferon.

at both concentrations of interferon used in these experiments. These results suggest that cytomegalovirus is more resistant to the antiviral action of interferon than any of the other representative viruses with which it was compared.

Interferon production. To determine if CMV induced the production of interferon *in vitro,* cultures of continuous line WI-38 or human embryo foreskin cultures were infected with CMV and followed until development of CPE. In different experiments supernatant fluids were harvested at varying time intervals (1-7 days after infection) and at different stages of involvement of the cell monolayer (25-60%) with typical CPE of CMV. This CPE correlates well with infection of the cells by CMV as evidenced by the presence of inclusion bodies, specific CMV fluorescence, and infectivity(9). Supernatant fluids were then harvested, acid treated, and assayed for interferon activity. As a control for the preparation and assay of human interferon, supernatant fluids from Chikungunya-infected WI-38 cultures were processed and assayed as a known interferon preparation. The results of two such experiments are summarized in Table I. In one experiment a minimal degree of antiviral activity was present at a 1:2 dilution of the supernatant fluid. Sufficient quantities of this preparation were not available, however, to characterize the inhibitor as interferon. In subsequent experiments, no such inhibitory activity was detected. These data suggest that cytomegalovirus is a relatively poor inducing agent for interferon production in cells of human origin in tissue culture.

Suppression of interferon production. A number of viruses have been shown to have the capacity to inhibit interferon production *in vitro*(10-13). To define further the relationship of interferon and CMV, the effect of this virus on interferon production was determined in human foreskin cultures. Replicate cultures were infected with CMV. After 48-96 hours, when CPE was first noted as discrete scattered foci and the monolayer of cells was still 99% intact, cultures were challenged with Chikungunya virus (CV) or UV-irradiated Newcastle Disease virus (NDV). In these experiments, inactivated NDV was utilized specifically to permit delineation of the effect of cytomegalovirus on interferon production in the absence of virus replication. Supernatant fluids were harvested at 24 hours and assayed for interferon activity. The results of two of these

TABLE I. Failure of Cytomegalovirus to Stimulate Interferon Production.

	Exp No. 1			Exp No. 3		
	Dilution	No. of plaques	% Reduction	Dilution	No. of plaques	% Reduction
Supernatant fluid from Chikungunya virus-infected cultures	1/10	0	100			
	1/20	5	91			
	1/40	19	67			
Supernatant fluid from cytomegalovirus-infected cultures	1/2	25	57	1/2	76	5
	1/5	58	0	1/4	74	7
	1/10	60	0	1/16	69	13
Control		58	—		80	—

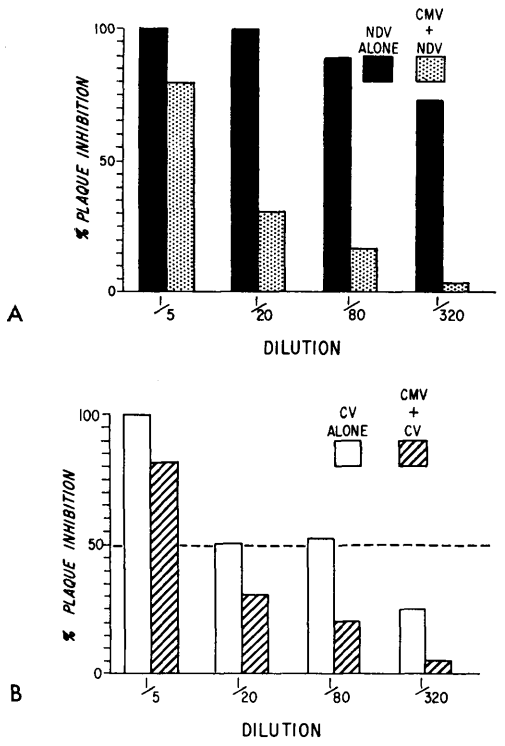

FIG. 3. Interferon levels in supernatant fluid from human foreskin cultures infected with: (A) Newcastle Disease Virus (NDV); (B) Chikungunya Virus (CV). The percent plaque inhibition by 4-fold dilution of preparations from Cytomegalovirus (CMV)-infected cultures is compared with control, uninfected cultures.

experiments are presented in Fig. 3. The presence of CMV significantly reduced the production of interferon following infection with either NDV or CV. These data suggest that CMV also has the capacity to suppress interferon production stimulated by other viral agents.

The possibility that CMV interferred with NDV adsorption was eliminated by the following experiments. Replicate cultures of CMV-infected or control human foreskin cells were exposed to 2 ml of MEM containing approximately 5×10^6 pfu/ml of NDV. An aliquot of NDV, maintained under identical conditions, was included in the protocol to control for thermal inactivation of virus. Samples were harvested from 3 cultures in each group at 15-60 minute intervals and assayed for unadsorbed virus by the hemadsorption plaque technique. In 3 experiments, no significant interference with adsorption of NDV could be demonstrated in cultures infected with CMV. In the experiment summarized in Fig. 4, 63% of the inoculum of NDV was adsorbed in the culture infected with CMV in comparison with 70% adsorption in the control culture. Over the 2-hour period of this experiment there was negligible thermal inactivation of NDV. These results suggest that CMV acts at an intracellular site in suppressing NDV-induced interferon production.

Sensitivity to interferon in vivo. Petralli and coworkers(14) have demonstrated that children develop detectable levels of circulating interferon between the 9th and 12th days after inoculation with live attenuated measles virus. The evidence that during the period of this interferonemia children are transiently resistant to smallpox vaccination has been interpreted to indicate that this interferon may alter the course of a virus infection in humans(15). To examine further whether cytomegalovirus was sensitive to the antiviral action of interferon under natural conditions of human infection, 3 children were studied who had been shown to be chronically infected with cytomegalovirus as

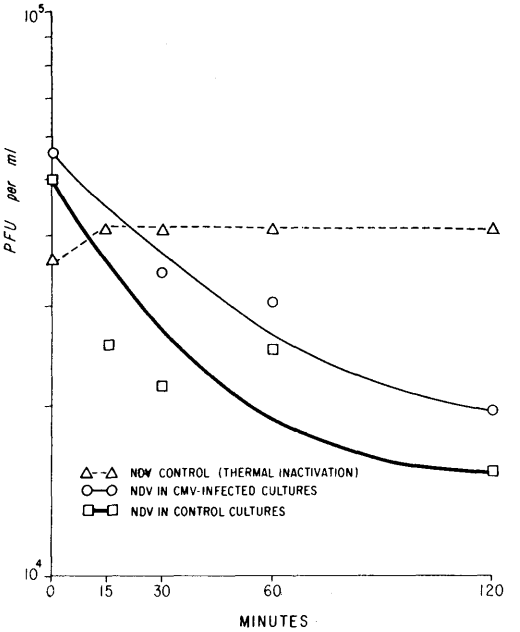

FIG. 4. Adsorption of NDV from supernatant fluid of control is compared with CMV-infected HFS cultures.

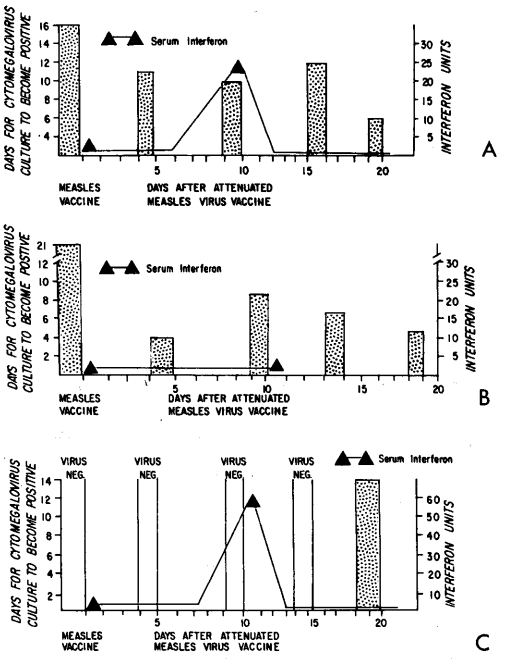

FIG. 5. Interferon levels, (▲—▲) expressed as units/ml, in serum of 3 children with chronic Cytomegalovirus (CMV) infection. Presence of CMV in urine samples collected during course of the study is represented by speckled bars.

evidenced by continued excretion of the virus in the urine. These children were inoculated with live attenuated measles virus vaccine after obtaining baseline blood and urine samples. Subsequent urine specimens were collected for viral assays on the 4th, 9th, 14th, and 18th day after vaccination. A second blood specimen was drawn on the 9th day to coincide with the expected peak in circulating interferon following live measles virus vaccination. The results of this experiment are summarized in Fig. 5. One child (Fig. 5, B) who was 8 months of age failed to develop any reaction to the vaccination or to produce detectable levels of interferon. This is probably explained by the presence of maternal antibody which is known to interfere with the response to attenuated measles vaccine and to prevent the induction of interferon. The other 2 patients developed significant levels of interferon, 1:25 and 1:60, respectively, in the serum. One of these patients (Fig. 5, A) continued to excrete virus throughout the experiments. The second child (Fig. 5, C) was even more interesting. Although she had previously been known to be chronically infected with CMV, repeated urine specimens during the course of the experiment were free of virus until the 18th day after vaccination when she was again found to be excreting cytomegalovirus. Although the interpretation of these data is limited by the number of patients in the series, there is no evidence that circulating interferon induced by attenuated measles virus has any suppressive effect on the multiplication and excretion of cytomegalovirus in human carriers.

Discussion. Infection with any of the viruses classified in the Herpesvirus group, including cytomegalovirus of human or murine origin, may be associated with the development of a long-term carrier state. The mechanisms which permit these agents to persist in the host have never been completely elucidated. That a normal immune response with neutralizing antibody does not necessarily accomplish the elimination of these viruses either *in vivo* or *in vitro* is widely recognized (1). In tissue culture *Herpesvirus hominis* infections are not eradicated by the addition of neutralizing antibody to the culture medium and *in vivo* individuals with recurrent herpetic lesions usually have significant levels of antibody in their serum.

More recently, interferon has been implicated in host resistance to virus infection both *in vivo* and *in vitro*(2-4). If interferon does, in fact, contribute to the process of virus elimination the capacity of a virus to circumvent the host interferon response may be one factor associated with chronic infection by that agent. Such a phenomenon could be explained on the basis of a relative lack of sensitivity to the antiviral action of interferon on the part of cytomegalovirus or the capacity of the virus to multiply in host tissue without inducing the production of interferon in host cells. One representative of the Herpesvirus group, *Herpesvirus hominis,* has been reported to be relatively resistant to the action of interferon both *in vitro* and *in vivo*(6,7). In addition, the virus of varicella-zoster appears to induce little or no interferon in infected cultures(16). The present work suggests that a similar situation

obtains with cytomegalovirus. Not only is this virus relatively resistant to the action of interferon, but it also is capable of multiplying in human cells with the induction of a minimal interferon response. It would seem reasonable to postulate, therefore, that these properties may be related to the persistence of this virus within an infected host.

In contrast to the present report, Henson and Smith(17) have found an interferon-like inhibitor in medium harvested from murine salivary gland virus (MSGV) infected cultures. Final yields of encephalomyocarditis virus (EMC) in their cultures treated with undiluted medium containing this inhibitor were reduced approximately one log when compared with control cultures. Similar preparations also effected a 60-75% reduction in EMC plaque counts. This inhibitor shared many characteristics with standard interferon although the preparations reported were not examined for species specificity, a direct inhibitory effect on the challenge virus, EMC virus, or for activity against a wide range of other viruses. This finding of relatively low levels of interferon-like activity induced by the related murine strain of CMV may reflect: (1) differences between human and murine strains of virus; (2) differences between the cytomegalovirus-host cell systems investigated; (3) a more sensitive assay system; (4) the presence of an inhibitor of EMC virus which may not fill all the criteria of interferon upon further characterization; or (5) the use of a higher inoculum of MSGV, 1×10^5 pfu as compared with the dose of approximately 10^3 $TCID_{50}$ of human cytomegalovirus used in this study. Further support for the concept of cytomegaloviruses as relatively poor inducers of interferon comes from the work of Osborn and Medearis(18). These workers have found that murine CMV similarly induces no detectable interferon in the organs of infected mice, and is unaffected by interferon both *in vivo* and *in vitro*.

Osborn and Medearis(18) also presented evidence that CMV-infected mice have a reduced capacity to respond to virus infection with the production of interferon. The data presented from our *in vitro* studies in cultures of human tissue suggest that the capacity to inhibit host interferon production in response to other viruses is a property of the human CMV as well. Other viruses have been recognized to have the property of stimulating infection by heterologous viruses and in certain instances this has been associated with the capacity to inhibit interferon production(10-13). The evidence that a virus which commonly results in a long-term chronic parasitic relationship in humans may also affect host response to other virus infections suggests a possible mechanism for synergistic action of cytomegalovirus and other human pathogens in carriers of this agent. Whether CMV infection actually alters the host response in humans has not been documented. Two out of the three children with chronic CMV infection reported in this study did respond to live measles vaccine with a detectable level of circulating interferon. The inhibition of interferon production *in vivo* reported by Osborn and Medearis(18), however, was observed only during the acute phase of CMV infection and therefore is not in conflict with the response noted in the children reported in this study. Further studies of the interaction of CMV with other viral agents are currently in progress.

Summary. Evidence is presented which indicates that human cytomegalovirus is relatively insensitive to the antiviral action of interferon *in vitro,* and that the rate of excretion of cytomegalovirus in the urine of a chronic human carrier was unaltered by circulating levels of interferon in the serum. In human cells in culture, CMV failed to induce the production of detectable levels of interferon. It is suggested that these phenomena may be related to the capacity of the virus to persist in the infected host for long periods. Human cytomegalovirus was also shown to have the capacity to suppress the interferon response of cultures of human tissue infected with other viruses. The implication of this finding for possible synergistic effect of cytomegalovirus with other viruses is considered.

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Studies on Immunological Tolerance to Soluble Proteins. I. Organ Distribution of Antigen in the Adult Mouse.* (32221)

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Following a neonatal injection of human gamma globulin (HGG) mice are rendered tolerant for at least 14 weeks(1). This state of immunological unresponsiveness has been thought to depend upon an interaction between the antigen and the early cell forms responsible for specific immune competence (2). Other studies have indicated the possibility of an extracellular regulatory mechanism for antibody synthesis(3). A specific state of immunological tolerance also can be produced in adult animals. HGG has been found to render adult C57 Bl/6 mice tolerant after a single inoculation of a relatively small dose of protein antigen(4).

Mice tolerant to HGG do not recognize HGG as a foreign material as shown by the exponential elimination of the human globulin from the mouse sera(1). The specific failure of unresponsive mice to form circulating antibodies may represent a failure of antigen uptake by the macrophages. A better understanding of the central problem of immunology, the mechanism of the immune response, can be gained by a better knowledge of the cellular basis for distinguishing between self and non-self. It seems that the degree of immunogenicity correlates with antigen localization(5).

The purpose of this investigation was to study the fate of the tolerance-inducing antigen in adult mice under different experimental conditions. The organ distribution of a low amount of antigen, known to produce specific immunological unresponsiveness, was studied in groups of previously untreated, tolerant, actively immunized and passively immunized mice.

Materials and methods. Animals: Inbred HS Swiss albino mice were obtained from Dr. B. R. Jennings(1). They were fed with a balanced diet and KI was added to the water bottles.

Antigens: Human gamma globulin (HGG) obtained from Pentex, Inc., Kankakee, Ill.,

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