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Virus-host cell interaction : in-vitro studies with canine distemper virus

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by
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TABLE OF ABBREVIATIONS

allS = acetone extracted horse serum

CDV = Canine distemper virus

CNS = Central nervous system

CSF = Cerebral spinal fluid

CHS = Dialyzed horse serum

DI = Defective interfering particles

DI-I'El', = Dulbecco's modified eagle medium

FCS Fetal calf serum

G1 Growth 1 phase

G2 = Growth 2 phase

H/S Horse serum

M = Mitotic phase

IIS = Multiple sclerosis

PBS = Phosphate buffered saline pH 7.4

R = Restriction point

RAD = A measure of the dose absorbed from ionizing radiation equivalent to 100 ergs of energy per gram.

RNA= Ribonucleic acid

SSPE = Subacute sclerosing panencephalitis

I N T R 0 D U C T 1 0 N

Natural history of measles and canine distemper infections

Canine distemper virus (CDV) was the virus primarily used in these studies, but it seems relevant to discuss the general properties and interrelationships of both measles and canine distemper viruses in view of their very close structural and antigenic relationships. Although, to our present knowledge, CDV does not infect man, measles of course does, and under proper conditions might be capable of causing delayed effects which could be similar to those observed as a result of canine distemper infection.

In their natural hosts, both measles and canine distemper viruses invade the respiratory system. Measles is known to infect the lung epithelia; indeed, measles virus can be isolated fro.,:1 patients with giant-cell pneumonia, which was at first thought to be a lung disease not involving any viruses, even when the characteristic measles rash is absent. This initial stage is follo.wed by an acute viremia, and the dissemination of the virus to many tissues of the body, including skin, nervous system, intestinal tract and renal tract. Cells of the lymphatic system are also susceptible to infection, and circulating leukocytes serve as a means of viral transportantion within the body. An important event in the development of measles infection in man is the occurence of marked leukopenia, a decrease in the number of white blood cells, 11 to 13 days after exposure. This depression in the number of white blood cells affects each one of the different classes of white blood cells , to a different degree. Monocytes seem unaffected, while lymphocytes and neutrophils are reduced in mumber, and eosinophils are virtually absent from the citculating blood at this time.

It is clear that measles virus infects both T and B lymphocytes. Osunkoya et al. (1974) initially reported that the infection is passive, meaning that no infectious particles are proGuce(l. It has been subsequently shown, however, that a small number of T and B lymphocytes are productively infected. McFariana (1974) has reported a suppression of helper T activity in experimental measles infection, and this activity appears not to be due simply to a reduction in the number of helper cells, but rather to some direct interference in their function. Huddlestone et al. (1980) conl"irmed the observation of Osunkoya et al. that passively infected lymphocytes can be stimulated to produce infectious particles by mitogens. Galama et al. (1980) reported that measles virus infection inhibited the acquisition of lymphocyte function, but that established functions were unaffected. Futheemore, others have observed that cells which are secreting antibody continue to do so after infection with measles, but cells which are infected at the same time as in vitro antigen challenge are almost completely suppressed, as measured by IgG and IgMI response. These authors suggest that the immunosupressive effect of measles is specific for the initial phases of antibody induction, and that cells which are commited to antibody synthesis are relatively unaffected.

Both measles virus and canine distemper virus cause encephalitis in their natural host. Although encephalitis in humans as a complication of measles infection frequently than does CDV encephalitis in the of.measles infection is so high worldwide that occurs far less than does CDV encephalitis is the dog, the incidence of measles infection is so high worldwide that even rare human diseases due to measles infection receive great attention. Abnormal electroencephalographic patterns are exhibited by a majority of acute measles patients suggesting that in most cases thaL the virus infects the nervous system. In roughly 0.1% of these cases the patient develops severe encephalitis, and many such patients suffer permanent disabilities. Of particular concern is the apparent increase in the incidense of measles encephalitis with age. Although different authors cite different absolute numbers for the incidence of encephalitis, all seem to agree th-at there is an increase in this incidence between infancy and adolescence.

One of the major development in the study of the measles virus was the dramatic observation that the brains of patients who died from subacute sclerosing panencephalitis (SSPE) showed the presence of nucleocapsids very similar to those of measles. Payne et al. (1969) were ultimately able to recover virus from the brains of SSPE patients by co-cultivating the SSPE brain cells with continuous cell lines. Subsequent studies using immunofluorescent staining showed that the recovered virus was measles or a measles-like agent. It is now widely accepted that SSPE represents a rare chronic infection with measles.

Several studies have suggested that at least half of all SSPE cases occur in individuals who contracted measles before the age of two years, suggesting that the state of development of the hosts nervous system may play a role in the pathological storage Of measles virus nucleocapsids. The onset of SSPE may follow the acute infection by anywhere from two to thirty-five years, with the average interval about six to eight years. The distinguishIng characteristic of SSPE brains, as revealed at autopsy, is the presence of inclusion bodies which havt been shown to contain viral nucleocapsids.

It has been shown that the reason for the accumulation of nucleocapsids in the infected brain is the lack of the viral M protein which is believed to play and important role in the final assembly of the viral particle.

It is clear that the age and stage of nutrition of the host, the immunosuppressive effects of the infection itself, and other as yet unidentified host factors, may play a role on both the localization of the virus and the eventual outcome of measles infection.

Studies On multiple sclerosis (MIS) suggested that the disease might involve an infectious agent. Some studies indicate the involvement of measles or measles-like agent. Althouth there is no direct evidence for the involvement of measles or measle-like agent. Although there is no direct evidence is overwhelming. 1) The finding of high titers of anti-measles antibodies in both serum and the CSF of some, but not all MIS patients. 2)The demostration of impaired cellular immune responses against measles virus in these patients. 3) The similarity in the patterns of demyelination which is seen in MS patients and that in dogs which have contracted CDV encephalomyelitis. 4) The tentative demonstration of measles virus genomic material in the brains of MS patients.

Complex and variable neurological signs are a common feature of canine distemper virus. The central nervous system may be involved in the acute stage of distemper, or it may follow the acute stage by years. Canine distemper virus causes acute demyelination of the brain nerve axons. The localization of the virus in the brain does not seem to be random and some preference of the virus for certain brain sides has been observed. The chronic infection is characterized by panencephalitis, inclusion bodies, and demyelination. Work by other investigators suggest that, in the dog, CDV enters the CNS via infected lympocytes.

Experimental animal models

The best animal model of morbillivirus CNS disease is in rodents. Parhad eL al. (1980) reported the infection of hamsters following inhalation of measles virus. By using a neurotropic strain of measles they were able to show the existence of measles antigens in the lungs and brains of these animals.

A number of laboratories have reported a progressive neurological disease which develops in hamsters and mice some months after intracranial inoculation with measles. Some investigators have reported the occurence of hydrocephalus as a complication of subacute encephalitis following the inoculation of hamsters with virus from Hela-derived cultures. Experimental work in mice has shown that, although the hamster neurotropic strain (HNT) of measles virus causes rapidly fatal encephalitis in newborn mice, the same virus produces abortive infections in animals over seven days of age. The immunosupression of weanling mice with cyclophosphamide did not alter the mortality rate from measles encephalitis suggesting that the age differences in susceptibility, might not be due solely to the development of immune competence. The differences in the development of acute infection between the suckling mouse and the newborn mouse appears to be dependent on the maturation of the cells of the central nervou System rather than on either virological or immunological factors.

In their investigations of CDV encephalitis in hamsters, Cosby et al. (1981) were able to correlate the plaque size of Onderstepoort CDV clones with the onset of encephalitis. Large (1-2mm) plaque isolates caused rapid encephalitis, whereas the small (<0.51mm) plaque isolates caused encephalitis only after a delay of from weeks to months.

Lyons and his colleagues (Lyons et al.,1980) demostrated that following intracerebral injection of mousebrain adapted CDV, some survivors showed symptoms of subacute CNS disease 3-9 months after infection. Tnese investigators were able to demostrate that the strains of mice most susceptible to acute infection were the ones least likely to develop chronic infection.

In addition, Lyons and his colleques observed the development of an obesity syndrome in some of the mice which had recovered from acute canine distemper encephalitis. The major characteristics of this syndrome included hypertrophy and marked hyperpiasia of the adipocytes, hyperinsulinemia and hyperplasia of the pancreatic islets, and decreased levels of brain catecholamines.

Canine distemper and infection

In order to examine the relationship of canine distemper virus to these long-term effects on the host organism, I have looked closely at the behavior of the virus in vitro for clues to its growth and its pathogenesis in vitro.

Canine distemper virus is so similar to measles that in fact the primary difference between the two viruses is only their host range. For practical purposes CDV may well be called dog measles (Choppin, personal communications). Canine distemper is a negative strand RNA virus that is surrounded by a lipid bilayer envelope. Two major polypeptides are attached on this envelope pointing, towards the outside of the virus (figure 1).

These are the H and F proteins; H stands for hemaglutinin and is responsible for the attachement of the virus on the hosts membrane, and the F that stands for fussion and is the one responsible for penetrading the hosts membrane. The 1.1 (matrix) protein is found immediatly inside the virus and it is believed to play an important role in the assembly of the viral particles. In the center of the virus we find the NP (nucleoprotein) polypeptide around which the genomic RNA is wound. Two other polypeptides the P and L are associated with the NP protein and they are believed so play a role in the assembly of the viral particles. CDV replication does not involve a DNA intermidiate nor does it require the entry of the virus in the hosts nucleus.

As was mentioned earlier, in its natural host, the dog, CDV causes acute and chronic encephalomyelitis. The chronic infection, often called "old dog encephalitis", has received widespread attention due to the occurence of demyelinating lesions which resemble those occuring in the human disease multiple sclerosis. Cook & Dowling (1978) have commented extensively on the possible involvement of CDV in multiple sclerosis. These chronic effects of a CDV infection are quite important because they manifest themselves long after the occurence and subsequent recovery of the host from the infection. Also, this chronic CDV infection bears strong similarities with measles encephalitis. The impications of this are very important since approximately 50% of all children that get measles show signs of some brain involvement Some rare diseases resulting from a measles are SSPE and MS. These are the result of a chronic measles infection which involves the replication of the virus in the brain as long as 10 years after the initial and subsequent recovery of the host from the infection. For these reason the experimental infections of the brain are potentially important.

In addition to its natural host, CDV is capable of productive infections in mice, hamsters, and several other laboratory species. Because of the close antigenic and structural relationship between CDV and measles, the experimental infection of mice with CDV may shed light on naturally occuring infectious in humans. in our laboratory, we have demostrated that the experimental infection of mice with strains of CDV can induce not only acute encephalomyelitis, but also chronic pathological events which manifest themselves long after the resolution of the initial infection. One immunocytochemical study of CDV infected mouse brains has shown that the virus is not uniformly distributed in these brains nor it is randomly distributed. The virus seems to favor certain areas of the brain over others (see appendix A).

In addition to the chronic infections in vivo, CDV causes both acute and persistent infections in a wide variety of cell lines. It has been reported that maintenance of persistent infections vitro is due to the involvement of both the presence of virus variants and a defect in the ability of the infected cell to replicate the virus efficiently. Defective interfering particles (DI), impicated in the establishment and maintenance of persistent infection in vitro have been isolated from a number of persistently infected cell cultures. Therefore, the released virus contains a large number of noninfectious particles that interfere with virus replication. DI particles have not, however, been demonstrated to play any role in chronic in yiy.Q infections.

Secuence of events When the infectious inoculum is added to a cell culture, the virus attaches on the hosts membrane and manages to penetrate it within a few hours. Viral replication will begin a couple of hours later with the H and F polypeptides been made first
As soon as they are made they move towards the hosts membrane which then they penetrate from the inside awaiting further assembly of the virus. The NP protein is then formed and the viral genome after being replicated is attached on it. The L and P proteins along with the 11 protein assist in the final assembly of the viral particle which takes place at the membrane of the host where the H and F protein are waiting. When the assembly is complited the viral particle will detach from the hosts membrane taking with it a piece of this membrane.

In general, viral infection often involves change of host cell growth control. The relationship between growth control and cancerous cell transformation has resulted in the extensive study of RNA tumor viruses by many investigators. These viruses, as their name implies, alter the growth control of the host resulting in an uncontrolled growth of the host that ceases only upon the exhaustion of the nutrients available to the cell. The relationship between growth control and viruses which do not cause tumors has not been widely studied.

Growth control of normal cells Before we can discuss the changes in host-cell growth control as a result of a CDV infection, we have to review some aspects of growth control in normal cells. The cell cycle starts with the G1 phase, in which the cells, whose biosynthetic activi- ties have been greatly slowed during mitosis, resume a high rate

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The S phase begins when DNA synthesis starts, and ends when the DNA content of the nucleus has doubled and the chromosomes have replicated. The cell then enters the G2 phase, which ends when mitosis starts.
cytokinesis. During the early part of the M phase the replicated chromosomes condense from their extended interphase condition and are easily seen in the light microscope. The nuclear envelope breaks down and each chromosome undergoes precisely orchestrated movements that result in the separation of its pair of sister chromatids as the nuclear contents are divided. Two new nuclear envelopes then form, and the cytoplasm divides to generate two saughter cells, each with a single nucleus. This process of cytokinesis terminates the 11 phase and marks the beginning of the interphase of the next cycle.

In multicellular organisms, cells cannot keep going through the cycle constantly and for this reason the cell has to maintain a number of control mechanisms. Whether or not *a mammalian cell* will grow and divide is determined by a variety of feedback control mechanisms. It has been determined experimentally that a certain protein has to accumulate in the cell in sufficient quantity at the end of the G1 phase, before the cell can grow and divide (Rossow et al., 1979). In fact the point of no return known as the resrtiction point (R), occurs late in G1. Once the cell has passed this R point, it is committed to completing the S, G2 and M phases

In tissue culture, other mechanisms controlling cell division include the availability of space in which a cell can flatten and the secretion of specific stimulatory and inhibitory factors by cells in the immediate environment. These factors may play a role on the phenomenon of contact inhibition exhibited by cells in tissue culture. This mechanism is responsible for

lowing down considerably the metabolic processes of the cell as soon as the cell comes in close contact with neighboring cells.
Lectins, which are a class of plant proteins that specifically bind on glycoproteins, have been shown to assist the cell in escaping contact inhibition when they are added into a cell culture. The involvement of a surface cell-receptor in this mechanism of contact inhibition has been assumed, but the precise mechanisms remain uncertain.

Another exogenous substance that alters the growth control of the host when added to a cell culture is colchicine. Since colchicine is a microtubule disruptor, the cytoskeleton of the cell has been extensively implicated in the control of cell growth and division. Colchicine has been shown to assist the cell in escaping contact inhibition. When contact-inhibited cell cultures receive a small amount of colchicine they regain a normal metabolic rate and continue to grow. The cytoskeleton involves many components the most important of which are the actin filaments and microtubules which are labile structures. They can be formed rapidly from a pool of soluble subunits in the cell and rapidly disassembled when no longer needed. Another major component of them cytoskeleton is the intermediate filaments, which are ropelike polymers of fibrous polypetides that have been presumed to play a structural or tension-bearing role in the cell. Actin filaments, microtubules, intermediate filaments, and their associated proteins are regulated by unknown mechanisms to produce changes in cell shape and various cell movements. In addition, the cytoskeleton seems to- organize the cytoplasm by binding various membrane-bound organelles and soluble proteins. Microtubules emanating from the cell center determine the distribution of intermediate filaments and appear to be responsible for establishing and maintaining cell polarity. The organization of a cell's cytoskeleton can be influenced by that of its neighbors either through intercellular junctions or by the extracellular matrix, and it can be passed on to its daughter cells when the cell divides.

In the course of examining the parameters governing CDV infection, both in vivo and in vitro, I have obtained results which suggest that the course of infection of the virus in vitro is intimately related to the growth regulation of the host cell, as evidenced the effect of a serum starvation and stimulation on infected cells in tissue culture. The effect is associated with a non-dialyzable component or components in serum.

Virus strains

Plaque-purified substrains of Onderstepoort strain canine distemper virus and of Edmonston strain measles virus were used. The original virus cultures were obtained from the laboratory of Dr. P. Choppin.

cell culture

Vero (African green nonkey kidney) and Hela cells were propagated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, and 100 units/ml penicillin. DMEM is a specially designed medium that contains essential nutrients necessary for cell growth in tissue culture. Cells are grown until they become confluentin 37 C incubator in an atmosphere of 5% co2. When cells become confluent they have to be transfered into a new dish where they will resume their normal growth. This passage is accomplished by washing them with 2ml of 0.25% trypsin (1:250, Gibco), a proteolytic enzyme which breaks the proteins that assist the cell in remaining attached to the tissue culture dish. The trypsin is then removed from the flask 30 seconds later, and 2ml of fresh trypsin is added for 3-5 minutes. Following trypsinization the cells are suspended in fresh DMEM supplemented with FCS. The suspension of the cells in FCS is necessary because the serum contains molecules that will inactivate trypsin thus allowing the cell to reattach onto the new substrate. Cells were grown in 75mm flasks (Corning) and routinely

passed at 1:6 ratio; a single flask of cells yields six new flasks.

Infection

Cells were transfered from the 75mm flasks into 60mm tissue culture dishes using trypsin-EDTA. The trypsin used (as above) contains 0.02,10 EDTA,, which is a chelating agent that removes divalent ions from a solution. Forty-eight hours after the cells had been plated onto the dishes (Corning) the medium was removed and 50 virus inoculum was added to each dish along with one ml of DMEM without serum. The dishes are incubated at 37 C and frequently tilted to ensure proper mixing of the inoculum and covering of all cells. Ninety minutes later 5ml of DMEM with horse serum were added to each dish. The addition of serum supplemented DMEM after incubation was found to be absolutely necessary for Hela cultures but not for Vero cultures.

Plaque Assay

For the purpose of assaying a particular viral batch used in an experiment, Vero cells are grown in 50mm dishes until confluent.. They are infected as described before, except that when the inoculum is removed 90 minutes after infection, the culture is overlayed with DMEM/Agar. The DMEM/Agar mixture if prepared by autoclaving 2% agar in distilled water, cooling it to 60C, and then mixing it at a 1:1 ration with double-strength DMEM (without
serum.) at 37C. Eight milliliters of this medium are placed on each dish and the dishes are allowed to cool at room temperature until agar re-gels and then are returned to the incubator. In order to make the observation of the viral plaques in these dishes more convenient an overlay of agar containing 0.0025% neutral red, a vital stain, is placed onto the dishes. The cell monolayers will absorb this stain making the visualization of the plaques more dramatic.

Cloning of Virus

In order to obtain a genetically homogeneous stock of virus for the experiments, the plaque assay dishes are used for the cloning of the virus. A Pasteur pipette is used to remove the agar above a plaque. This piece of agar is then placed into confluent Vero cultures and the virus is allowed to grow. The virus is then harvested from these Vero cultures and stored at -70C. This method yields a homogeneous stock of virus since every plaque in a plaque assay dish is in principle a result of one parent viral particle. And since care is taken to remove only one plaque at a time from these dishes, the resulting progeny is quite homogeneous.

Growth of virus in mice

The Onderstepoort strain of canine distemper virus is one adapted for growth in mice. The infectious inoculum is injected in young mice intracranially, using a 26 guage hypodermic syringe. No more that 10ul of this inoculum can be **injected into** a mouse at one time. It is therefore convenient to use a mechanical devise that delivers a precise amount of inoculum every time. When the mice develop the usual signs of infection, lethargy and tremor, usually from four to seven days, they are sacrificed by cervical dislocation. Their brain are then removed and homogenized in 10ml Hank's buffered salts per gram of brain weight. The homogenate is then centrifuged for 20 minutes at 10k X g. The supernatant is collected an stored at -70 0 C. where it remains stable indefinately.

Experimental Infection

Mice can be injected incracranially with infectious fluid at an age of 3 to 6 weeks old. The injection is made using a 26 gauge hypodermic syringe, at the mid dorsal part of the head, a little Do the left of the mid-sagittal plane. The choice for this injection side is a very carefull one in order to avoid penetration of the sagittal sinus, which is a large blood pool along the midline immediately beneath the skull. a blood pool. Penetration of the sagittal sinus would result in excessive bleeding and subsequent death of the animal.

Detection of virus by Co-Cultivation

Sometimes is not possible to detect the presence of virus in an infected brain by adding the brain homogenate into confluent Vero cultures. In this case it might be possible to **recover** some virus by the technique of co-cultivation. A small piece of a CDV-infected mouse brain is minced under sterile conditions and placed into a tissue culture dish. Some of the cells from the brain will migrate out of the tissue and grow in the dish. At this point some Vero cells are added to the same dish and by coming in close contact with the infected brain cells they also become infected indicating the presence of the virus. Occasionally several passages of this culture are required before any cytopathic effect can be observed in the Vero cells.

Immunofluoresenc

Hela and Vero cells were grown on microscope coverslips, and infected with CDV as described before. The microscope coverslips have to be treated with collagen (Flow Laboratories) for 2 hours before cells can be placed onto them. This is done in order to provide the cells with an appropriate substrate for attachment. This is a necessary step since cells do not attach well on glass coverslips. After the treatment with collagen the coverslips are placed into tissue culture dishes. These coverslip-containing dishes have to, in turn, be sterilized. Sterilization takes place by exposing the dishes to gamma radiation for 20 minutes at 15000 RADS. The irradiated dishes are then ready for tissue culture. For staining the coverslips were washed in acetone for 30 seconds followed by three 5-minute washes in PBS. They Were then incubated with a high-titer human anti-measles serum at 1:100 dilution for 90 min., washed three times in PBS and then stained with an anti-human Ig fluorescein-conjugated antibody for go minutes. Coverslips were viewed under a fluoresence microscope.

E X P E R I M E N T S &R E S U L T S

Experiment 1

In our hands Hela cells incubated with CDV exhibit little or no syncytia formation and lysis of cells, which is the usual cytopathology of infection shown by infected Vero and other cultures. That these Bela cultures were successfully infected was shown by immunocytochemical studies, and it was observed that although the conventional cytopathology was absent, the infected cultures formed foci of replicating cells. When these cultures were observed, through the microscope, dense foci of replicating cells could be

seen scattered throughout the culture. These cultures were sLained immunocytochemically for CDV antigen and it was observed that many of these foci were brightly stained indicating the presence of large amounts of viral antigen.

Such cultures were found to be quite sensitive to serum stimulation following infection with CDV virus. Although Hela cells were usually grown in DMEM supplemented with 10% fetal calf serump 'it was found that DMEM supplemented with 10% horse serum, increases tnreefold the number of foci appearing in the infected cultures as result of infection.

In order to establish that this formation of foci in infected Hela cultures was independent of the specific tissue culture plasticware, focus formation was observed in dishes of three major manufacturers. The same focus formation in all three types of tissue culture dishes indicated that this phenumenon was independent of the type of substrate used.

During the course of these experiments it was-observed that the precise timing of horse serum stimulation was very important for the formation of foci. Hela cultures were infected with CDV and they received DMEM/HS approximately one and a half hours post-infection. Cultures were incubated for four days, at which point the medium from the dishes was removed and fresh DMEM/HS was added to the dishes. Twenty-four hours following the addition of fresh DMEM foci of replicating cells appeared throughout the infected dishes (figure 4).

experiment 2

In order to confirm the casual observation that horse serum yielded different results from fetal calf serum, in the cytopathology of infected cultures, we infected a number of Hela cultures, under controlled conditions. One and one half hours after the infection, half the infected cultures received DMEM/FCS and the rest received DMEM/HS (5ml per dish). Four days later fresh DMEM with serum was added to the dishes, as above, and the Lures were incubated for 24 hours.

It was observed that cultures receiving DMEM/HS developed many foci of replicating cells, whereas cultures that received DMEM/FCS had only a third the number of foci exhibited by the DMEM/HS cultures. Curiously, the DMEM/HS cultures exhibited a number of floating cells, their number almost equalling the number of cells attached on the monolayer. Some of the floating cells were collected and replated: they attached to the sustrate, were viable, and capable of replication. Floating cells from ininfected dishes were also collected and replated but they were unsuccessful in attaching on the dish and thy died within 72 hours.

experiment 3

In order to examine the dependence of focus formation on serum concentration, Hela cells were infected with CDV, as before. Ninety minutes post-infection, infected cultures were divided into groups. Each group received a different concentration of DMEM/HS; 2%, 4%, 6%, 8%, AND 10%. Cultures were incubated for four days, at which point fresh DMEM1 containing the same concentration of horse serum was added to each dish.

It was noted that at a horse serum concentration of 6% infected dishes exhibit foci of replicating cells while uninfected dishes of the same horse serum concentration consisted of a single monolayer with no apparent increase in cell growth or focus formation (Table 1)
1)

Infected cultures with a horse serum concentration less than 6% did not exhibit focus formation. At horse serum concentration greater that 6% the infected cultures developed more foci, but

focus formation was also seen in uninfected cultures receiving the same *concentration of* horse serum. Thus, as expected the focus formation observed in not unique to infected cultures, but infected cultures *were sensitive to* serum stimulation at a lower serum concentration than uninfected cultures.

Experiment 4

For a very preliminary characterization of the horse serum factor(s) which may be responsible for the phenomenon of focus formation in the infected Hela cultures, we tested the activity of dialyzed serum and acetone extracted serum. The serum was dialyzed against PBS across a 120OOmw cutoff membrane. The dialysis was performed at 40C for 24 hours with three changes of PBS. For the acetone extraction, 40ml of horse serum were added to 10 volumes of cold acetone with stirring. The precipitate was collected and *washed* three times in 10 volumes of cold ethanol. The precipitate was again collected, resuspended in PBS and dialyzed three times against PBS across a 12000mw cutoff nembrane.

The activity of the dialyzed horse serum was tested as usual, by adding it into infected Hela cultures, one and a half hours post-infection. Cultures were stimulated by refreshing the dHS four days later. Twenty four hours later focus formation was observed among both the infected and uninfected cultures (Table 2).

The acetone extracted horse serum was also tested on infected Hela cultures four days post-infection. Twenty-four hours later focus formation was observed in both infected and uninfected cultures, as with the dialyzed HS. Of the three preparations compared, native horse serum was found to have the highest activity while acetone extracted HS has the least. Nonetheless, aHS still showed the ability to stimulate cells.

Experiment 9

During the previous experiments it was noted that focus formation occured when MIIEM/serum was refreshed at four days. In order to determine the period during which the cells are susceptible to stimulation, Hela cells were infected with CDV, as before. One and a half hours post-infection, cultures received 5ml of D'IM/HS. The cultures were then divided into four groups. Each group received fresh D14E!.*,/HS at a different time interval -after the infecti on; 24 hours, 48 hours, 72 hours, 96 hours and 120 hours.

The addition of DMMAI/HS at 24 or 48 hours post-infection was ineffective in initiating focus formation in the infected cultures. Focus formation was apparently maximum at 96 hours. In 'addition, it was observed that infected cultures couls be incubated without any addition of fresh DME14/HS for up to twelve days. No foci formed during this period. If DMEIVHS is added, even as?late as day 12, focus formation appears within approximately 24 hours after the addition. If the infected cultures are left beyond day 12, no futher stimulation is possible, and no focus formation can be initiated.

In keeping with previous reports on the heterogeneity and variability of the viral population generated by a morbillivirus infection, we have found noticeable variability in the in vitro consequences of CDV infection. Some of this variability is apparently independent of the substrain of virus

In Vero cells, CDV primarily causes the usual previously described cytopathology; plaque formation and subsequent lysis of cells. In Hela cells, CDV causes the formation of foci of cells apparently analogous to those observed by Gould (1974) in his original studies on measles virus. Whereas Gould was studying the behavior of plaque-purified measles, I observed the relationship between the occurence of foci and the precise conditions of tissue culture. Apparently without syncytia formation, CDV renders infected cells more sensitive to horse serum stimulation. Mien Hela cells are stimulated with horse serum approximately four days post-infection they form a number of foci of replicating cells. The concentration of horse serum necessary to elicit focus formation is lower for infected cells than for uninfected cultures. By contrast, I have been unable to observe this pnenomenon using fetal bovine serum, and presume that the serum factor of factors necessary for focus formation may not be present or be present at very low concentration in fetal calf serum. It might be imagined that the phenomenon is an indirect one; infected or dying cells may release growth factors into the medium. However, when these foci of replicating cells were died using immunofluoresence they were stained brightly, indicating the presence of a large amount of viral proteins. I observed

at the timing of the refreshment of DME2.1/HS plays an important role in the virus-host interaction and that the appearance of Increased susceptibility to serum stimulation coincides with the replication cycle of the virus. It seems clear that the presence of the virus sometimes makes the cells more susceptible to serum stimulation. I would like to suggest that perhaps this susceptibiliLy may arise from disruption of the cytoskeleton. In studies

c~f growth control, microtubule disruptors, most notably colchi-

cirle, have beer: used to alter the cell's sensitivity to serum -stimuication (McClain and Edelman, 1980). To my mind, it is plausible ttat the disrurion of' the cytoskeleton which accompanies CDV infection may hav-_ similar results. However, the data presentea here ao not allow us to rule out other possible mechan**1ISMIZ.** such as the Interference of the virus with the expression br proper funcLioz; of a cell-surface receptor. In addition, it re-mains unclear wnether the conditions optimal for focus formation in Hela cultures are or are not selecting for possible virus varia4td which are unrepresentative of the original virus stock. Measles virus recovered by Gould in his original study was apparently genetically distinct from the starting cultures. Gould has isolated different progenies of measles virus by picking out individual viral plaques from his infected cultures. But he was not able to infect new cultures by only using the viral progeny recovered from a single plaque, and only upon mixing the progeny of a number of different plaques was he able to infect new cultures, suggesting a possible mechanism of complementation. Continuing studies of the virus recovered from the.infected Hela

cultures should resolve this question.

Although Gould was the first to observe the occurence of foci in measles-infected cultures, he provided no explanation to this effect. I have determined that certain horse serum factor(s) are responsible for this phenomenon in infected cultures. and that the phenomenon is not observed in the absence of serum. The active presence of canine distemper virus inside the cell renders the host more susceptible to serum, stimulation. This interaction between the virus and host-cell alters the host's growth control mechanisms causing the formation of dense foci of replicating cells.

APPENDIX A

Acute Experimental. CDV Encephalitis in *Mice*

Brain reFi	Observation
Rhinencephalon	
HippocamPus	bright staining of pyramidal cells
Telencephalon	
cerebral cordex	random diffuse
Basal ganglia	random diffuse
DiencephalOll	
Hypothalamus	uncertain
I-'IeT,encephalon absolutely negative	
Cerebellum	
Myencepbalon	
Brainstem	bright staining
	ventral to fourth ventricle

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