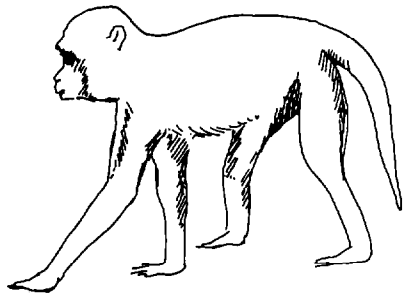
Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Section I

The Use of Cell Cultures for Presumptive Identification of Viruses

P. papio
(Guinea baboon)



P. cynocephalus
(Yellow baboon)

Chapter 1

The baboon story: origin of the use of cell culture selectivity for differentiating viruses *

In the early 1950s, the attack rate of paralytic poliomyelitis was at its peak in the United States. The development of a safe and effective vaccine against poliomyelitis was essential for its prevention and control. In dire need of a cell culture system appropriate for the production of poliovirus vaccine, Dr. Joseph Melnick suggested I test the sensitivity of baboon kidney cell cultures to poliovirus infection. At that time (1955), I was a neophyte in the field of cell culture (originally called tissue culture) and virology, and was ignorant of what a baboon looks like. Nevertheless, armed with much curiosity, I started my career by hands-on learning how to prepare kidney cell cultures from two baboons, *Papio papio* (guinea baboon) and *Papio cynocephalus* (yellow baboon) (see Fig. 1), the former from West Africa and the latter from south Central Africa, and testing the sensitivity of their kidney cells in culture to viral infection.

Cells from each baboon kidney yielded thousands of culture tubes. Even after inoculation of the 3 types of serially diluted poliovirus suspensions, a large excess was on hand. Not wanting to waste the 'left-over' cell cultures, I asked Dr. Melnick if I could test additional enteroviruses available in the laboratory at that time.

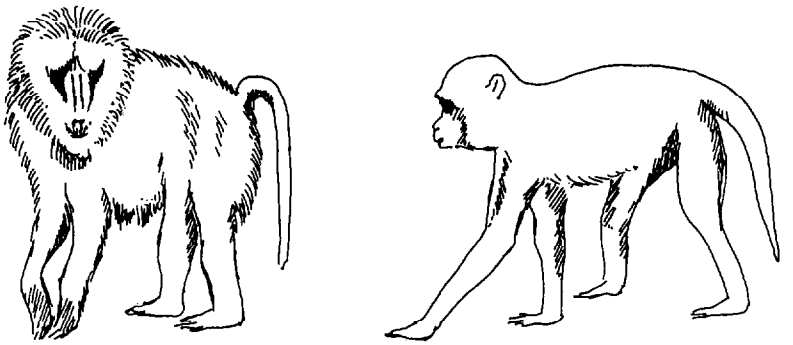


Fig. 1. The baboon story.

* This chapter is dedicated to Dr. Joseph Melnick on the occasion of his 80th birthday.

These included 5 types of Coxsackie B virus, Coxsackie A9 virus, and 14 types of echoviruses, all were tested by serial dilutions. Inoculated culture tubes were microscopically examined daily for evidence of cytopathic effect (CPE). Microscopic reading for CPE in thousands of culture tubes proved to be very tedious; however, curiosity continued and careful examination for evidence of cellular changes was initiated 24 h postinoculation. To my great surprise, monolayers in culture tubes derived from both baboons showed distinct CPE when inoculated with the 3 types of poliovirus and 5 types of Coxsackie B viruses. On the other hand, cultures inoculated with Coxsackie A9 and most of the 14 types of echoviruses induced cellular changes only in cells derived from *Papio papio*, but were completely absent in cultures derived from cells of *Papio cynocephalus*.

As a trained bacteriologist, I instantly thought of the application of the principle underlying the differentiation of Gram-negative bacteria by the sugar fermentation test; i.e. *E. coli* ferments both dextrose and lactose while *Salmonella* and *Shigella* sp. ferment dextrose only. A parallel concept was formulated for virus identification and its application in the rapid differentiation of virus isolates as in the case of polio-, Coxsackie A and B and echoviruses. Indeed, as is the case for the differentiation of Gram-negative bacteria by the sugar fermentation test, so it was, more or less, for the differentiation of human enteroviruses by selective sensitivity of cell cultures.

At the 1956 Federation Meetings in Atlantic City, the concept of selective sensitivity of cell cultures for virus differentiation was presented (Hsiung et al., 1956). Though the concept was considered revolutionary, it was viewed with hesitation and skepticism by the audience, including the well-known virologist, the late Dr. J. Smadel. At that time, viruses were mostly identified and characterized by serologic methods.

Over the years, we have taken advantage of the use of the selective sensitivity of cell cultures to identify viruses for rapid presumptive diagnosis of viral infection. Due to scarcity of baboon cells, kidney cell cultures derived from other non-human primate species, and many other cell lines, have been investigated. Similarly, they were found to have varying degrees of selective sensitivity to viral infections. Cultures of primary rhesus monkey kidney cells were the most sensitive cell system for the isolation of human enteroviruses.

In order to search for a suitable substitute for the rhesus monkey, a list of non-human primate cells were tested against enterovirus infections, as in the case of the baboon cells (mentioned above). These included: gibbon (*Hylobates*), cynomolgus (*Macaca iris*), white-crowned Mangabey (*Cercocebus torquatus*), sooty (*Cercocebus fulginosus*), green (*Cercopithecus aethiops*), diana (*Cercopithecus diana*), moustached (*Cercopithecus cephus*), spot-nosed (*Cercopithecus nictitans*), mona (*Cercopithecus mona*), sykes (*Cercopithecus mitis*), and patas (*Erythrocebus patas*). Among the new world monkeys tested were: capuchins (*Cebus capucinus* and *Cebus cirrifer*), black lemur (*Lemur macaco*), ring-tailed (*Lemur catta*), and thick-tailed bush baby (*Galago crassicaudatus*).

As a result, a monkey species was singled-out; an African monkey, i.e. the patas monkey kidney cells not only favored a high-titered yield of poliovirus, but was also consistently resistant to infection by most echoviruses and Coxsackie A9 virus.

Thus, kidney cell cultures derived from rhesus and patas monkeys showed results similar to those noted in the two baboons; i.e. rhesus = *P. papio*, and patas = *P. cynocephalus* separating polioviruses together with the 5 Coxsackie B viruses, from most echoviruses together with Coxsackie A9, once again, analogous to those differential media used in a bacteriology laboratory. The two kinds of monkey kidney cells 'rhesus' and 'patas', have been used for presumptive diagnosis of human enteroviruses into two groups (Hsiung and Melnick, 1957).

In spite of the patas cells distinct differential susceptibility/sensitivity to enteroviruses, the search for the substitute of patas monkey kidney had to continue because patas monkeys are even more rare than rhesus. It was at this time that a development took place; cell lines derived from human cancer origin then became fashionable. A cell line, Hep-2 (derived from human larynx carcinoma), was given to our laboratory by Sloan Kettering Institute in New York with the idea to search for a virus cytocidal for cancer, but innocuous for normal human cells. After a series of experiments, it turned out that Hep-2 cells mimic the patas cells in that they are insensitive to infection by most of the echoviruses, although they are highly sensitive to the 3 types of poliovirus and all the Coxsackie B viruses.

Differing sensitivities of cultured cells are advantageous for rapid viral diagnosis, particularly for the human enteroviruses, to date. Isolation of poliovirus and other non-polio enteroviruses, especially from stools, necessitates a differential host system(s), not only to obtain an early diagnosis so essential for the proper management of the patient, but also to shorten the period of anxiety of the patient and family members.

The key to rapid viral diagnosis which I believe deserves emphasis, is the proper choice of cell culture systems for a particular virus. Thus, very often, when I visit a clinical virology laboratory, I do not ask what kind of viruses they are isolating, rather what kind of cell culture systems they are using, considering that individual viruses can only be isolated in their respective susceptible cell culture systems. Investigators are therefore obliged to be open-minded in so far as the use of selective sensitivity of cell culture systems for detecting viruses of unknown etiology is concerned.

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Chapter 2
Dispute of the classification of echovirus-10

Originally, echoviruses consisted of a group of agents that induced cytopathic effect in cell cultures inoculated with stool samples from children with or without specific clinical disease. Thus, 'ECHO' virus represents enteric cytopathogenic human orphan viruses, the agents of which are in search of their disease entity. As suggested by the Enterovirus Committee (1959), the term ECHO is to be used as a temporary repository for viruses awaiting final identification/classification.

In the midst of a wild search for a differential cell culture system for the entire enterovirus group, cell cultures derived from a variety of primates and non-primates were tested for their susceptibility/sensitivity to enterovirus infection. Let me recall from the previous chapter that guinea baboon and rhesus monkey kidney cell cultures are highly susceptible/sensitive to all enteroviruses tested, while yellow baboon and patas monkey cells showed relatively selective sensitivity to the enterovirus infection; such that 3 types of poliovirus and all group B Coxsackie viruses are patas-sensitive, while Coxsackie A9 and most echoviruses are patas cell-resistant. Our attention was caught by echovirus 10, which induces a cytopathic effect in kidney cells of a new world monkey, the lemur, the only one of several new world monkeys tested that was sensitive to human enterovirus infection.

Subsequent extensive search included non-primate kidney cells, such as domestic animals, e.g. calf, pig, dog and cat, and laboratory animals, such as rabbit, guinea pig and hamster for their susceptibility/sensitivity to enterovirus infection. Of all the 14 types of echoviruses tested, only echovirus 10 induced CPE in the non-primate kidney cultures. Echovirus 10 infectivity titers obtained in non-primate cell cultures were essentially the same as in their primate cell culture counterparts. At this point, it was evident that echovirus 10 displayed a host cell range entirely distinct from other human enteroviruses. It became clear that echovirus 10 was a virus of distinct biologic properties and should not be included in the echovirus group (Fig. 2) (Hsiung, 1958).

I presented our findings at the VI International Congress for Tropical Medicine and Malaria in Lisbon, Portugal in 1958 (Hsiung, 1958) and proposed that echovirus 10 should be deleted from the enterovirus group. The late Dr. Albert Sabin was in the audience. Since echovirus 10 was originally isolated in his laboratory (Ramos-Alvarez et al., 1956), he was skeptical about the proposal. However, in 1959, the name of reovirus-1 was proposed for echovirus 10 by Dr. Sabin (Sabin, 1959).

In 1962, the first Diagnostic Virology Workshop was conducted at the Department of Epidemiology and Public Health at Yale University School of Medicine.

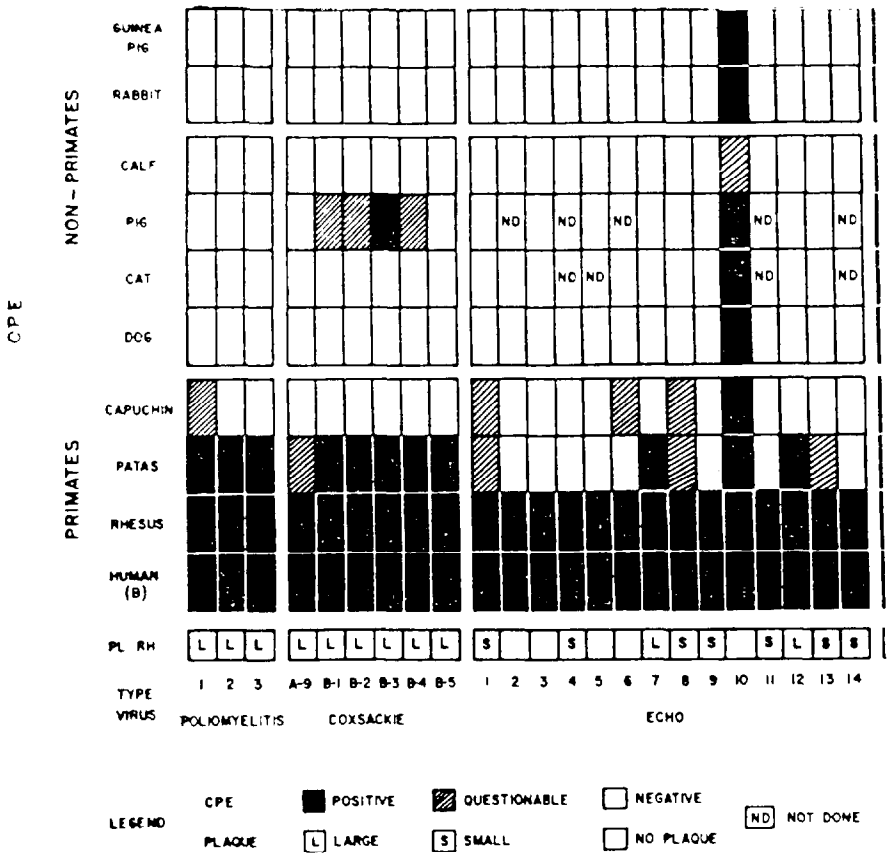

Fig. 2. Host range on selectivity of human enteroviruses (poliomyelitis, Coxsackie and echovirus) infections. Modified from Hsiung (1961, Yale J. Biol. Med. 33, 360).

Benchwork in the course included identifying viruses given as unknowns, utilizing a variety of techniques. One of the unknown viruses, echovirus 10 (reovirus-1) showed brilliant green inclusions in the cytoplasm of infected monkey kidney cells following acridine orange staining. It was a mystery since echovirus 10 is an RNA virus and should induce red inclusions in the cytoplasm after acridine orange staining. Peter Gomatos, M.D., who was pursuing a Ph.D. at the Rockefeller University was one of the participants at the workshop. At that time, Peter was studying the molecular biology of reovirus, and found that reovirus had a double-stranded RNA, a property that is distinctly different from other RNA viruses. Since our unknown echovirus 10 induced green inclusions in the cytoplasm of infected cells following acridine orange staining, it established that echovirus 10 is a double-stranded RNA virus. Apparently, double-stranded RNA would stain green in cytoplasm instead of red as single-stranded RNA following acridine orange staining — what a miracle!

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Chapter 3
The magic of cell culture sensitivity for detecting genital herpes

Genital infection due to herpes simplex virus (HSV) was one of the most common venereal diseases in the United States during the 1970s. The epidemic of genital herpes has aroused great public concern. Genital herpes is different from other common venereal diseases in that it has no cure. During the course of primary infection, the virus travels via sensory nerves to the dorsal root ganglia where it can establish a latent state, and can be reactivated at any time. In addition, if a pregnant woman has active infection at the time of delivery, she can transmit the virus to her newborn child, and the infection in the neonate is often fatal. Furthermore, an epidemiologic association between infection with HSV-2 and cervical cancer has been noted. Thus, it is important to make an accurate diagnosis of HSV infection in the genital tract, especially in pregnant women.

One of the rare features of HSV is that it is one of the few human viruses that will replicate well in non-human as well as human cells. Thus, HSV replication often obtained in non-primate cells including primary rabbit kidney, guinea pig embryo fibroblasts and mink lung (ML) cell line *faster* than cells derived from primates, including human cell lines, like MRC-5, and monkey kidney cells such as Vero cells, a kidney cell line derived from African green monkey kidney cells. If an insensitive cell system is used for isolation of HSV from a clinical specimen containing only a few infectious virions, failure to isolate HSV is a probability. This is illustrated in the following case.

One day, I received a phone call from Dr. Edward Kraiselburd of the University of Puerto Rico (San Juan, Puerto Rico)... "Dr. Hsiung, you told me to use mink lung (ML) cells instead of Vero cells for the isolation of HSV from the genital tract of pregnant women in my survey study. I noted that 100% of virus isolations were obtained in both ML and Vero cells inoculated with specimens taken from women with genital lesions. On the other hand, specimens taken from women without lesions only induced CPE in ML, but not in Vero cells. However, all isolates were identified as HSV-2, and they all showed similar thymidine kinase (TK+) enzyme activities. Why is it that HSV-2 isolates obtained from asymptomatic women do not grow in Vero cells upon primary isolation? Yet, I cannot find any difference between the isolates obtained from the two sources". My immediate answer was that in the asymptomatic women, the number of infectious HSV in their genital tract is low, thus a highly sensitive cell system is necessary in order to detect the small number of

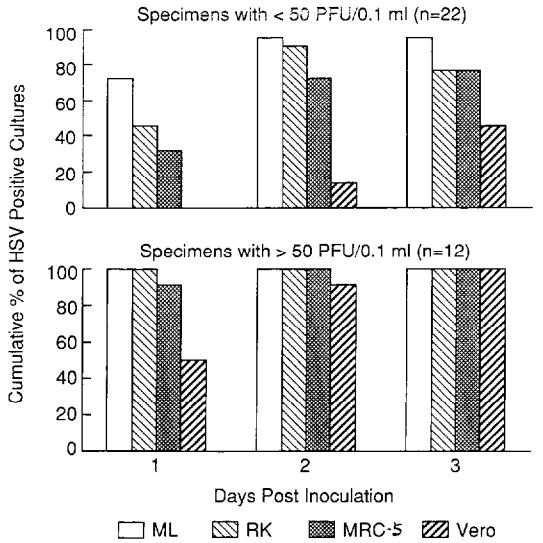

Fig. 3. Isolation of HSV from clinical samples using mink lung (ML), rabbit kidney (RK), human fibroblast (MRC-5) and Vero cells.

infectious virus present. I do not think that there is any difference genetically between viruses isolated from the two groups of patients.

Our experience with diagnostic work on genital herpes has taught us a 'secret' in order to obtain rapid and accurate diagnosis of genital herpes. If an insensitive cell culture system is used for the detection of HSV in clinical specimens containing only a low virus concentration, failure to isolate HSV is a possibility. Therefore, the 'magic' lies in using a sensitive cell culture system in order to have a rapid amplification of the virus for recognition.

Fig. 3 shows the comparative sensitivities of 4 cell culture types in the recovery of HSV from clinical specimens (Zhao et al., 1987). Note that when a clinical specimen contained less than 50 infectious HSV virions/0.1 ml (top), there was no evidence of virus infection in the Vero cells, yet 72.7% of the specimens cultured showed virus infection in a sensitive ML cell line even on the first day postinoculation. If, on the other hand, clinical specimens contained high titered virus, i.e. greater than 50 virions/0.1 ml (bottom), Vero cells detected only 50% while ML cells detected 100% of the positive cultures on day 1 postinoculation. In parallel, the group of pregnant women with lesions represents high-titered HSV in their vaginal swabs, while the group of pregnant women without lesions represents the low-titered HSV in their vaginal swabs; thus, detection of virus from patients without visible lesions was possible only when the most sensitive cell system is used.

The 'magic' revealed is the need for an appropriate sensitive cell culture system to ensure rapid and accurate viral diagnosis, no matter what the virus is being aimed at. I cannot overemphasize the value of this 'magic'.

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Chapter 4
Unexpected rewards of using multiple cell culture systems

It is often the unexpected findings that make discoveries! The two incidents narrated in this chapter attest to the concept that open-minded use of cell culture systems leads to unexpected findings.

In the first incident, Dr. Marie L. Landry, a young physician in our NIH Clinical Virology Training Program at that time (1980–1981), obtained a throat swab from an 8-year-old boy (PiCh), with a diagnosis of herpangina (Landry et al., 1981). Bearing in mind that human viruses, in general, only replicate well in cells of human or non-human primate origin, with a few exceptions, Landry, being a new investigator in clinical virology inoculated PiCh samples into all cell culture types available at that time in our laboratory, including HELF (human embryonic lung fibroblast), HEK (human embryonic kidney), HP (human placenta), Hep-2, RhMK (rhesus monkey kidney), and GPE (guinea pig embryo) (see Fig. 4). Inclusion of GPE was because

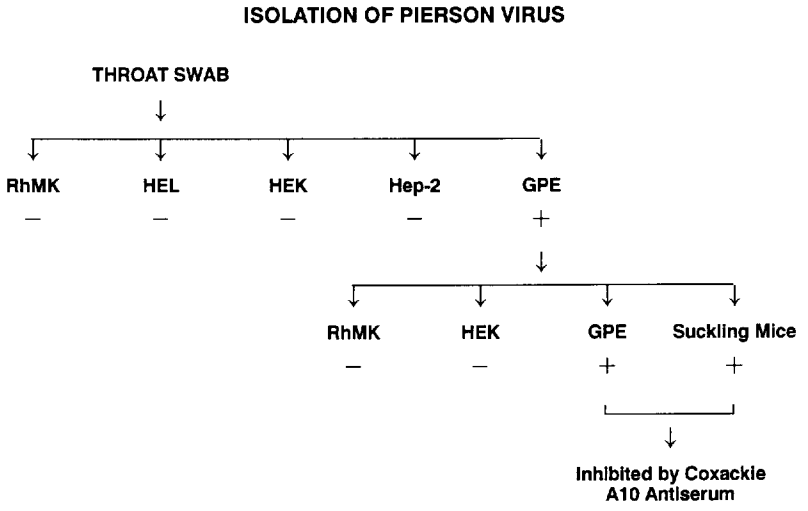

Fig. 4. Flow chart of the original discovery that Coxsackie A virus induces cytopathic effect in cultured guinea pig embryonic cells. RhMK, rhesus monkey kidney cell; HEL, human embryonic lung cell; HEK, human embryonic kidney cell; Hep-2, human larynx carcinoma cell line; GPE, guinea pig embryonic cell.

of its availability in the laboratory at the time for our research projects on guinea pig viruses. Lo and behold (!), an extensive virus-induced cytopathic effect was observed *only* in GPE cells within 3 days postinoculation; no evidence of cellular change was noted in any of the other cell culture types, including human cells similarly inoculated. After one passage of PiCh virus in GPE cells, HELF cells became susceptible, and it took as many as 8 passages in GPE cells before HP cells supported PiCh virus replication.

Because of no prior knowledge that any human virus will induce CPE in GPE cells, identification of PiCh virus posed a problem. Electron microscopic examinations of negatively stained preparation of infected GPE cell culture supernatant and thin sections of infected GPE cells revealed a picornavirus. Subsequently, when the supernatant of infected GPE cells was inoculated into newborn suckling mice, all mice died within 2 days. Hindlimb paralysis was noted 2 days postinoculation. Skeletal muscle histopathology revealed diffused hyaline degeneration, loss of striation, and mononuclear cell infiltration. It was then that PiCh virus was suspected of being a Coxsackie A virus. Serology by the neutralization test identified PiCh virus as Coxsackie A10 virus. Antiserum against Coxsackie A10 inhibited both plaque formation and CPE induced by PiCh virus. Other antisera against Coxsackie A1–9 and 11–24, group B Coxsackie types 1–6, poliovirus types 1–3, and echovirus 9 did not neutralize PiCh virus.

The aftermath of the above unexpected finding was a study using GPE cells for known isolates of Coxsackie A viruses from clinical specimens. It turned out that GPE cells are as sensitive and susceptible as suckling mice for the primary isolation of several types of Coxsackie A viruses. GPE cells became a handy alternative to suckling mice, which are not always available when needed, for routine use in the laboratory.

The open-minded inclusion of GPE cells in the primary isolation procedure rewarded us with unexpected findings. First, GPE cells support the replication of Coxsackie A viruses comparable to that of suckling mice; second, its availability makes it feasible for routine primary isolation of Coxsackie A viruses from clinical specimens when infant mice are not available. What's more? GPE cells made the isolation of PiCh virus (Coxsackie A10) possible, which could have been missed since most human and non-human primate cells are resistant to Coxsackie A virus infection, as illustrated in this case.

The second incident relates to another unexpected finding, i.e. GPE cells are susceptible to certain types of adenovirus (Landry et al., 1987). Initial pathology findings of the patient's lung showed herpesvirus inclusions, probably due to CMV. Only after electron microscopy were the inclusions recognized as actually being adenovirus crystals (see Chapter 16). In addition, CPE was noted in GPE cell monolayer inoculated with stool filtrate of the above patient. Isolation of adenovirus in GPE cells was totally unexpected, considering that adenoviruses are usually species-specific. Human adenoviruses replicate best in human cells, and poorly or not at all in cells derived from other animal species. The isolate was subsequently identified as adenovirus type 2.

As in the first incident, the unexpected finding prompted us to study the susceptibility of GPE cells to other known adenovirus prototypes (Fong et al., 1987). It was

found that GPE cells favored only the replication of adenovirus types 2 and 5, but poorly type 7, and negligible for types 3, 8 and 31.

Be open-minded! It is always possible that new cell lines will be found sensitive for certain viruses, if they are to be tested.

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Chapter 5
Mysteries of mixed viral infections

Concurrent infections with two or more viruses are commonly seen especially in immunocompromised hosts. Since latent infections with adenovirus, herpesvirus (HSV, CMV, VZV, EBV), papillomavirus and polyomavirus are often noted in healthy individuals, these viruses can be reactivated intermittently in the respiratory or genital tract with or without overt disease. It is often a mystery to detect mixed viral infections without special manipulations.

In dual viral infections, recognition of two enterovirus infections by plaque morphology (see Chapter 7) is easily recognized, but when an inoculum of cerebrospinal fluid (CSF) of a 7-month-old baby induces CPE in Hep-2 cells and plaque formation in rhesus monkey kidney cell monolayer, it posted a mystery (Hsiung, 1965). Further testing revealed that the isolate which induced CPE in Hep-2 cells did not form plaques upon subculturing in rhesus monkey kidney cells under agar overlay, and the isolate which formed plaques in rhesus monkey kidney cells did not induce distinct CPE in Hep-2 cells. It was only after several passages of each virus isolate in its respective cell line that the Hep-2 cell isolate was identified as adenovirus type 7, and the isolate in rhesus monkey kidney cells was identified as Coxsackie virus B1. Thus, more often than not, successful recovery of more than one viral agent from clinical specimens is obtained by using multiple cell culture systems.

Almost 30 years later, Mori et al. (1993) reported a dual virus infection of poliovirus type 2 (vaccine strain) and adenovirus obtained from the CSF of an infant without evidence of an immunodeficiency syndrome. They used 3 cell lines, MA-104 (a green monkey kidney cell line), MRC-5 (human embryonic fibroblast), and Hep-2 (human larynx carcinoma cell line) cells.

In both of the cases described above, isolation of adenovirus would have been missed were it not for the use of Hep-2 cells in the primary isolation process. Enteroviruses would have been the only viral agents isolated from the cerebrospinal fluid and would be considered as the cause of the disease of the patient involved.

In the early days of diagnostic virology, fewer cell culture systems were available. Currently, most of the laboratories are using multiple cell systems in their isolation attempts. These include MRC-5, A549, RhMK and RK or GPE cells (Fong and Landry, 1991). Thus, two or more viral agents have been isolated from single clinical specimens of patients and require separate techniques for detection. In general, a certain cell culture type displays a sensitivity for a certain virus only to a large extent, and to a limited extent, sensitivity for more than two viruses; for example, MRC-5 for cytomegalovirus, RhMK cells for influenza viruses, and A549 for adenovirus

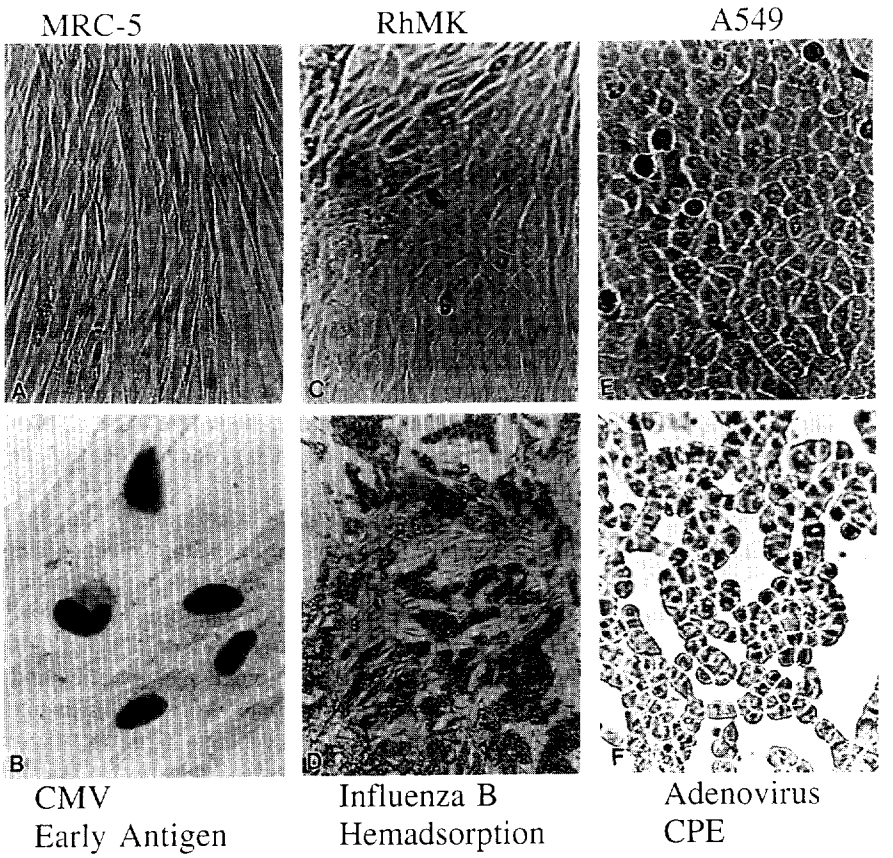

Fig. 5. Illustration of a mixed infection using 3 cell systems and recognition of the 3 viruses by 3 separate techniques. A and B: cytomegalovirus early antigen by immunofluorescence. C and D: influenza virus by hemadsorption. E and F: adenovirus by cytopathic effect.

(Fig. 5). In addition, recognition of each virus requires separate techniques. For example, cytomegalovirus induces early antigen in MRC-5 cells and can be detected by IF using monoclonal antibody; the presence of influenza virus in RhMK needs the addition of RBC for hemadsorption and adenovirus induced CPE in A549 cells often delays the observations (Hsiung, 1993).

Mixed infection remains a mystery since no one would expect such a finding when one starts to look for an unknown etiological agent of an infection or disease. There is no exception. In the winter of 1992 during Dr. Buck's virological survey of marine animals, a sample taken from the tongue lesion of a killer whale was submitted for virological studies; a mysterious incident occurred. After several passages of the isolate in primary African monkey kidney cell cultures, a few herpes particles were seen in one of the samples fixed for electron microscopy (EM). However after subsequent passages, two distinct cytopathology effects were noted when the samples were inoculated into two cell systems, the African green monkey kidney cells

(AGMK) and a dog thigh tumor cell line (A-72 cell). A rounded cell CPE occurred in AGMK cells, but extensive syncytial cell formation occurred in the A72-cell line. Since herpesvirus was suspected of causing the tongue lesion, infected monolayers from both cell cultures were fixed and embedded for electron microscopy. Although herpesvirus particles were observed in the AGMK cell, unexpectedly, retrovirus particles were found in the A-72 cells. In order to confirm the later observation, reverse transcriptase (RT) activity was examined from supernatant of the infected A-72 cell culture. Indeed, it was positive for RT activity in the presence of manganese. In this case, the mixed infection would not have been recognized without using the two cell systems, i.e., AGMK cells and A-72 cells, in the passages of the isolate. Identification of the two distinct viruses was made possible by the use of electron microscopy: a herpesvirus-like particle with cytomegalovirus-like morphology in the AGMK cells, and foamy virus morphology (retrovirus) in the A72 cells were evident.

This reminded me of a previous occurrence in our laboratory during early 1970, while we were searching for viral agents in normal bovine serum, a common component of culture medium. For several months, we had negative results in examining numerous lots of bovine sera using both cell culture isolation method and directly electron microscopy. In order to test the validity of our method of examination, we added known viruses — in this case parainfluenza type 3 (a paramyxovirus), and a bovine herpesvirus, infectious bovine rhinotracheitis (IBR) virus to 100 ml of calf serum. This mixture was distributed to two investigators without prior knowledge of the mixture. One milliliter was removed to test for the presence of infectious virus and 99 ml was concentrated for examination by electron microscopy. After 1 week of examination, according to Dr. Fong's EM result, virus particles of a paramyxovirus and also herpes particle were found in the calf serum sample. However, to my great surprise, only the herpes virus induced CPE was noted in the bovine kidney cell culture tubes inoculated with the known mixtures of calf serum samples, but the parainfluenza type 3 virus was never recovered by the second investigator, Dr. Swack (Swack et al., 1975). It was then realized that the lot of calf serum used contained a high titer antibody to parainfluenza virus type 3, thus preventing its recovery in the cell culture method, although the virus particles can be recognized under electron microscopic examination. What a miracle! From this experiment we learned a lesson that calf sera which contains antibodies to parainfluenza viruses should not be used in culture medium for clinical specimens. It was therefore suggested that culture medium should be free of calf serum for isolation of influenza and parainfluenza viruses.

Because of the evident restrictive selectivity of a cell type for a certain virus, the use of multiple cell culture systems is necessary and rewarding. It matters, therefore, that a cell culture system can amplify small amounts of virus and also possess a broad spectrum of support for viral replication, but detection of each virus may require separate techniques. The search for a universal cell culture system remains a challenge for cell culture enthusiasts. It is up to the current day and future generations of diagnostic virologists to make this dream a reality.

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Chapter 6
Recycling discoveries on 'presumptive identification' of human enteroviruses

As a researcher in basic virology, I had no prior experience in clinical medicine. In July 1960, at the suggestion of the late Dr. Robert Green, I took over the responsibility for the Clinical Virology Laboratory at the Yale-New Haven Hospital, I was then appointed as the Director of the Diagnostic Virology Laboratory at the Hospital by the late Dr. John Paul, with an attending physician, Dr. Fred Kantor, appointed by Dr. Paul Beesen, then the Chairman of Internal Medicine, to assist me with the clinical duties.

A clinical episode occurred on June 2, 1961 when a 28-year-old female was admitted to the Yale-New Haven Hospital 7 h after a sudden onset of bilateral pain in the upper abdomen. The rapid onset of incapacitating pleuritic pain with normal chest findings in a young healthy woman were strongly suggestive of pleurodynia, a disease commonly associated with Coxsackie group B virus infection. On the day of admission, as part of routine procedure, nose, throat and rectal swabs were taken from the patient by the attending physician, and submitted for virological studies. Blood was also withdrawn for serology.

In the laboratory, each specimen was inoculated into 3 tubes each of rhesus monkey kidney and patas monkey kidney cell cultures. Forth-eight hours postinoculation with the rectal swab specimen, cellular changes became visible in the rhesus, but not in patas cell cultures. A backtrack to Chapter 1 will tell us that patas monkey kidney cells are sensitive to polio-Coxsackie B viruses, but resistant to Coxsackie A9 and echoviruses. Based on the differential sensitivities of rhesus and patas monkey kidney cells, the clinical suspicion of a Coxsackie B virus causing the patient's pleurodynia symptoms was in question. From our previous study (Chapter 1), a presumptive diagnosis was suggested that the patient had an echovirus rather than a Coxsackie B virus infection. A preliminary report was therefore made that an echovirus was isolated from the patient and that the virus isolate was decidedly not belonging to the Coxsackie B group. Even the possibility of a mixed infection with a Coxsackie B virus was nil, since patas cells are usually highly sensitive to group B Coxsackie viruses. However, the attending physician was very skeptical at that time with the presumptive diagnosis based upon cell culture selectivity. Even after 7 days of observation, still no cellular changes were noted in patas cells.

Final identity of the virus isolated was echovirus 8 (Kantor and Hsiung (1962). Antiserum pools of poliovirus consisting of types 1, 2 and 3, Coxsackie virus B pool consisting of types 1–6, Coxsackie A9, and echovirus types 4, 9, 11, 14 and

16 did not neutralize the virus isolate. Complete neutralization was achieved only with antiserum against echovirus 8, and partially only against echovirus 1, thus the first case of demonstration that echo 8 cross-reacted with echovirus type 1. The preliminary presumptive diagnosis of an echovirus infection rather than a Coxsackie B virus infection was confirmed. This finding once again reminds us of one of the relationship 'virus and disease': a virus can cause a broad spectrum of clinical diseases, and conversely, a clinical disease can be associated with several different viruses.

The identification of echovirus 8 as the etiologic agent of the patient's symptoms consistent with pleurodynia was then subsequently confirmed by serologic testing of the patient's paired sera of which one was obtained on admission and the convalescent phase serum 22 days later. Her convalescent phase serum at a dilution of 1 : 160 significantly reduced the number of plaques of the virus isolate as well as the prototype echovirus 8, but not by the acute phase serum. Likewise, the convalescent serum showed an antibody titer of 1 : 10 against prototype echovirus 1, but not in her acute phase serum. For the first time, antigenic crossing between echovirus 1 and 8 was noted. As anticipated, no increase in antibody titer to any of the group B Coxsackie viruses was observed. The virological and serological workup results confirmed that echovirus 8 was responsible for the illness of the patient. Originally, echovirus 8 was an incidental finding in normal children, and was rarely associated with disease.

Were it not for the differential sensitivities of rhesus and patas cell culture systems to the various enteroviruses as described in Chapter 1, it would have been extremely difficult, if not impossible, to make the presumptive diagnosis in 2 days that the patient actually had an echovirus infection. Just imagine the bulk of serological tests that would have to be performed in order to search for Coxsackie B viruses which were considered to be associated with pleurodynia symptoms. Prior to the present finding, Coxsackie group B viruses have been commonly encountered with cases of pleurodynia. At the time, if only Coxsackie group B viruses and Coxsackie B probes were used to search for the etiology of the patient's illness, it would have probably been reported that the patient did not have a viral infection.

Over the years, we have taken the advantage of using selective cell cultures for presumptive diagnosis of virus infection (Hsiung and Melnick, 1957; Hsiung, 1962). The saying 'history repeats itself' is not inapplicable in this anecdote of 'recycling discoveries'. During our pioneering years of unrelenting search for a differential cell culture system for grouping enteroviruses, during the early 1950s, it was, as I recall, the observation that rhesus monkey kidney cells showed a broad-spectrum sensitivity to most, if not all the enteroviruses; in contrast, baboon and patas (Chapter 1) monkey kidney cells, to our surprise, displayed a partial differential biologic property. For the first time, without the use of serologic tests, enteroviruses were divided by the selectivity of kidney cell culture systems; i.e. cultures derived from *P. cynocephalus* (a baboon) were sensitive to 3 types of poliovirus and 5 types of Coxsackie B viruses, but were resistant to Coxsackie A9 and 14 types of echoviruses. Similarly, grouping into patas-sensitive and patas-resistant enteroviruses followed shortly; such as the 3 types of poliovirus and the 5 types of Coxsackie B virus belonging to the former,

Table 1
Recycling 'discoveries' on presumptive identification of human enteroviruses

Virus	Original proposal (1956)[a]			Subsequent reclaim (1990)[b]		
	RhMK	Patas MK	(Hep-2)	RhMK	Hep-2	RD
Polio 1–3	+	+	+	+	+	+
Coxsackie B 1–5	+	+	+	+	+	–
Coxsackie A9 and echo 1–14 selected types	+	–	–	+	–	+

[a] Hsiung, G.D. and Melnick, J.L. (1956) Differentiation of poliomyelitis, Coxsackie and ECHO (enteric cytopathogenic human orphan) viruses by plaque morphology and host cell susceptibility. Fed. Proc. 15, 1932. Hsiung, G.D. (1962) Further studies on characterization and grouping of Echo virus. Ann. New York Acad. Sci. 101, 413–422.
[b] Johnston, S.L.G. and Siegel, C.S. (1990) Presumptive identification of enteroviruses with RD, Hep-2 and RMK cell lines. J. Clin. Microbiol 28, 1049–1050.

and Coxsackie A9 and 14 types of echoviruses to the latter group. Since supply of baboons and patas monkeys was limited, the Hep-2 cell line was included in the early studies (Chapter 1). It turned out that Hep-2 cells mimicked exactly patas cells in their selectivity (Hsiung, 1962).

During the period 1980–1990, the booming computer age, history repeated itself. A computerized literature search can only go as far back as 5–10 years. Consequently, our early findings in the 1950s would have no access to the computer; a recycling 'discovery' occurred in the report by Johnston and Siegel (1990), who claimed that presumptive identification of enteroviruses into groups can be made using a panel of several cell culture systems (see Table 1).

In our early studies, we were searching for a single cell culture system that is most sensitive to enterovirus infection, especially for poliovirus vaccine production. Selective differential sensitivity to the different enteroviruses constitutes a by-product at that time, and we have suggested that in order to save antisera and laborious neutralization test, grouping enteroviruses can be facilitated by cell culture selectivity, a pioneer proposal. In fact, these laboratory findings have been applied to a clinical case stated above. Such a biologic property for presumptive diagnosis of enteroviruses for the past 30 years is remarkable. Due to the limited supply of antiserum pools for typing human enteroviruses, Johnston and Siegel were prompted to retrace our footsteps unknowingly. In both quests, the benefit of serologic testing was not invoked. So, what is new after all? History, indeed, repeats itself — discoveries are being recycled!

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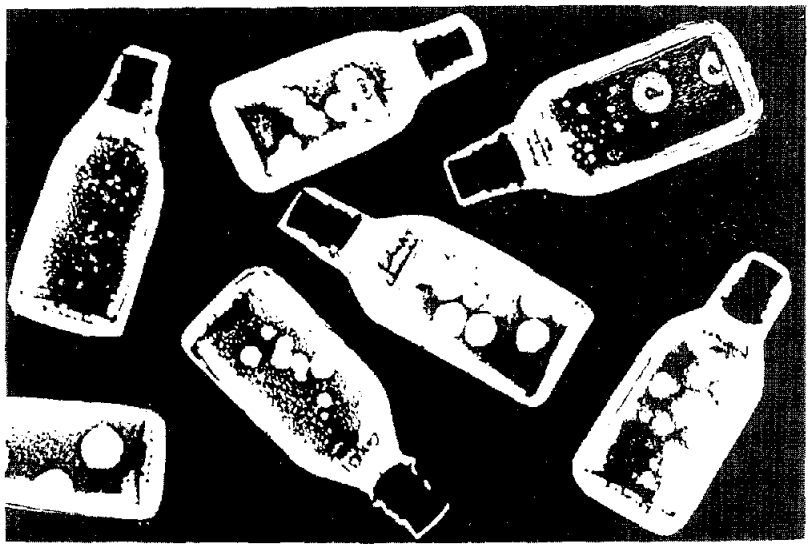
Section II

Bottle Cultures Enhance Human Virus Plaque Formation



Chapter 7
Enterovirus plaque formation in bottle culture: when the diagnosis is polio

The development of the plaque technique in monolayer cultures by Dulbecco (1952) advanced the versatility of tissue culture methods for studying animal viruses. However, in order to demonstrate plaques of slow-growing human viruses, it was necessary to adapt Dulbecco's technique to a closed system in stoppered prescription bottle cultures in which kidney cells were viable under agar for 2–3 weeks or longer. Thus, the need for the humidified incubator with CO_2–air mixture was eliminated. The elimination of a humidified incubator with a constant supply of a CO_2–air mixture was a great advance in the early 1960s since CO_2 incubators were not commonly available in most laboratories.

Because the original observations that human enterovirus induces plaques of various size and shape (Hsiung and Melnick, 1955), we were able to characterize the morphology and size of plaques produced by polioviruses, Coxsackie virus groups A and B, and echoviruses (Hsiung and Melnick, 1957), which I will discuss shortly, dissipated the mystery of poliomyelitis-mixed infections and also rapid diagnosis of poliovirus infection.

During the early 1940–1950s, the poliomyelitis epidemic was at its peak. Since mixed infection with other enteroviruses may have occurred from time to time, it was necessary to find out whether a patient was actually infected with poliovirus. In addition, the National Foundation for Infantile Paralysis supported a worthy cause: they offered free hospitalization and medical care for patients suffering from any form of poliomyelitis infection, not only paralytic disease. Since we reported that we can identify poliovirus and other enteroviruses by their plaque formation in bottle cultures, stool specimens suspected of containing poliovirus poured into our laboratory. The isolation of the poliovirus from a suspected case of poliomyelitis inf' .tion meant not only free medical care, but good medical care for polio patients. Viral isolates from stools of suspected cases of poliomyelitis were subjected to plaque testing. Poliomyelitis cases with mixed or dual virus infection were diagnosed without delay. Large, clear plaque, appearing 4–6 days postinoculation were recognized as poliovirus (Fig. 6). In the second week of the incubation period, appearing on days 8–10, small, irregularly shaped plaques, echoviruses, were found. If CPE were the only basis for virus identification, diagnostic pitfall would result. Dual virus infections with poliovirus and other enteroviruses would have been misdiagnosed altogether.

Amplification of enterovirus pathogens by plaque formation under agar overlay has been noted on many occasions. A good case in point is about a 34-year-old

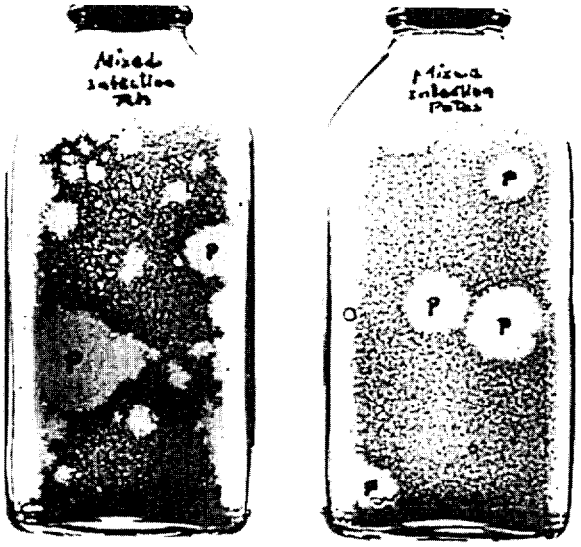


Fig. 6. Mixed infection with two enteroviruses: monkey kidney cultures inoculated with a fecal specimen containing two different enteroviruses. Left: rhesus monkey kidney culture showing both poliovirus type 1 (large circular plaques) and echovirus type 1 (small irregular plaques). Right: patas monkey kidney culture showing only poliovirus plaques.

male dentist who was admitted to the hospital with marked respiratory distress and complete heart block. This was a fatal case of only 24 h duration in the ward. Postmortem specimens (heart, spleen, liver, lung, muscles and intestines) in 10% suspensions were each inoculated into 3 of each of the following tube cultures: rhesus monkey kidney cells, green monkey kidney cells and Hep-2 cell cultures. After 2 weeks' observation, no CPE in any of the inoculated tube cultures was found. Frozen 'leftover' specimens were thawed and used to inoculate two bottles each of rhesus monkey kidney cells, then overlaid with agar medium. After 5 days, only the heart muscle inoculum yielded 5 and 15 plaques, respectively; plaques were medium-sized, round with clear centers. Virus from the 15 plaques did not replicate in Hep-2 cells in tubes which remained normal as in the primary isolation. Parallel subcultures replicated well in rhesus monkey kidney cells and produced distinct cellular changes in 48 h. This material provided the source of virus stock for identification which was finally identified as echovirus 9 by neutralization tests (Monif et al., 1967).

Compared with fluid culture, the plaque technique under agar overlay proved to be more sensitive for isolation of enterovirus when the number of infectious virus in the inoculum was too few to produce detectable CPE in fluid cultures. The formation of plaques in the primary isolation procedure served as an amplification in a localized area; therefore, an adequate amount of virus stock for identification purposes was possible.

A similar condition was obtained in the isolation of two strains of echovirus 22

from pneumonia patients. The clinical specimens of both cases failed to produce CPE in fluid cultures, but yielded enterovirus by plaque technique in rhesus monkey kidney cell monolayer under agar overlay; both enterovirus isolates were later identified as echovirus type 22. In each case, small, irregular, hazy plaques appeared after about 10 days of inoculation (Gabrielson and Hsiung, 1965). It is still difficult, even with the use of our current most sensitive technique, polymerase chain reaction (PCR), for the detection of etiological agents, to know which primer one should use without knowledge of a specific virus infection.

The usefulness of the plaque formation in bottle culture is summarized as follows: (1) it is sensitive for virus isolation particularly when the number of infectious virus particles are too low to be detectable by CPE in fluid cultures; (2) it serves for amplification of virus stock yield for identification purposes; and (3) it detects mixed virus infections rapidly by plaque morphology and size. Without the use of the plaque technique, many enterovirus infections and especially dual virus infections, would have been misdiagnosed.

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Chapter 8
The origin of DA paramyxovirus plaques: human or monkey?

The isolation of DA (David Anderson) virus, a paramyxovirus from a postmortem cardiac blood sample of a fatal case of acute hepatitis, was originally accomplished by plaque formation under agar overlay medium (Hsiung, 1959). Since then, its origin (human or monkey?) was disputed and has remained a mystery even to the present date!

The story of DA virus originated in Naples, Italy, when Dr. Robert W. McCollum went to investigate an epidemic of infectious hepatitis during early 1959. Along with serum samples collected from the outbreak, DA's postmortem specimens included whole blood from the heart, liver, stool and intestinal contents (Hsiung et al., 1962). In our laboratory, all DA specimens made into suspensions were inoculated into bottle cultures of rhesus monkey kidney (RhMK) monolayers, 6 cultures per sample, then overlaid with agar nutrient medium. In parallel, RhMK and HK (human kidney) cells in tube cultures were also inoculated with the same specimens and then replaced with fluid medium. Neither CPE was observed in any of the inoculated tube cultures with liquid medium nor plaques in bottle cultures inoculated with suspensions of liver, stool or intestinal contents. After 8 days of incubation, however, 5 of the 6 RhMK culture bottles inoculated with whole blood samples showed plaques that increased in size and number, giving an average titer of 16 plaques/ml of blood. None of RhMK and HK tube cultures originally inoculated with the whole blood in parallel with bottle cultures showed any evidence of virus-induced changes. When supernatant fluid of the RhMK and HK tube cultures originally inoculated with whole blood was subsequently tested in RhMK bottle cultures under agar overlay, clear plaques were again evident. Thus, direct isolation of DA virus was accomplished in RhMK cells and HK cells, although no evident changes in fluid cultures were seen, plaque formation under agar overlay medium was observed upon subculturing of the culture fluid. It was indeed a mystery for several months, because only virus-induced plaques were repeatedly obtained under agar overlay medium, yet no CPE was observed in parallel fluid cultures.

When single plaques were picked and subcultured into fluid culture tubes, no CPE was noted, even 7–14 days after subculturing, yet similar plaques always appeared in bottle cultures inoculated with serial dilutions of the supernatant of tube cultures. Interestingly, virus plaques only appeared when the inoculum was at higher dilutions, but not at lower dilutions. After several months of mystery, and serial passages

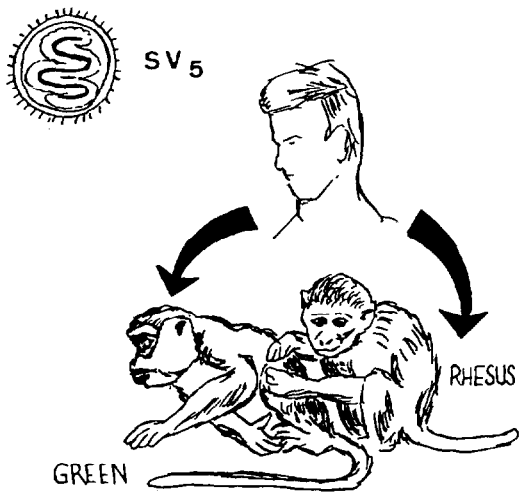

Fig. 7. Circulation of a paramyxovirus (SV_5) between human and monkeys.

of DA virus, it was found that DA virus induces the production of an inhibitory substance which rendered into autoinhibition of plaque formation (Hsiung, 1961). This inhibitory substance, in most of its properties, resembled interferon and related interfering substance. It should be noted that at the time, the 'hemadsorption' technique had not yet been discovered.

At about the same time, Schultz and Habel (1959) reported the isolation of a new myxovirus, the SA virus. Chanock et al. (1961) later identified that SA is related to SV_5, an isolate from monkeys (see Fig. 7), and that SV_5 is, in fact, serologically related to parainfluenza type 2, and has been claimed to be a monkey parainfluenza 2. Subsequently, further studies of DA virus showed that DA (isolated in our laboratory), SA (isolated by Schultz and Habel), and SV_5 (isolated from monkeys), are antigenically identical.

Due to such antigenic identity of the 3 viruses, the origin of DA virus was questioned; was it from the patient or from the monkey kidney cultures used for primary isolation? Testing serum samples from monkeys caught in the field and those already held in captivity showed that only 3% of field samples, and 25% of captive African monkeys had antibodies against DA virus (Atoynatan and Hsiung, 1969). From these data, it appeared that the DA virus is primarily a human virus, and that ecologically, humans may be infected with a virus identical to, or very closely related to, a virus which is acquired and widespread among monkeys when held under conditions of captivity.

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Chapter 9
Plaque reduction neutralization proved measles virus antibody 1 day after the rash

In general, measles virus-induced CPE in cell cultures is a slow process, usually requiring 2–3 weeks, especially with passaged cell line. For example, in Hep-2 cells inoculated with measles virus, it is necessary to constantly change the fluid medium in order to maintain the cells in healthy condition.

To our great surprise, the patas monkey kidney (PaMK) cells not only supported measles virus replication in fluid cultures, it also induced distinct small pinpoint measles virus plaques with clear centers and sharp boundaries in only 5–6 days (Fig. 8). Because measles virus plaques have never been observed prior to our observation, there is a question whether the minute plaques observed in PaMK cells under agar overlay were actually the same measles virus which have been customarily passed in Hep-2 cells.

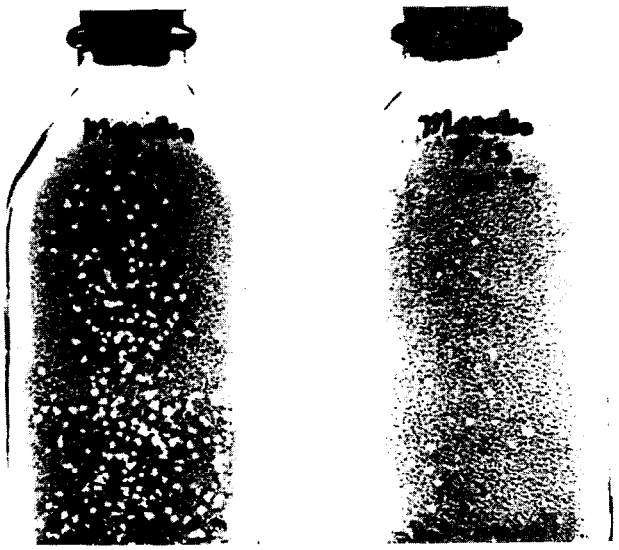

Fig. 8. Measles virus plaque formation in patas MK bottle cultures inoculated with the 13th passage of measles virus 10^{-1} and 10^{-2}, respectively. Plaque assay of measles virus in primary patas MK cell cultures, 6 days after inoculation.

We tested the 4th and 17th patas passages of measles virus, and the 7th Hep-2 passage measles virus stocks by plaque neutralization test. Yes! Immune serum against measles virus produced in a monkey neutralized both PaMK and Hep-2 cell-adapted measles viruses (Hsiung et al., 1958).

Further, we proceeded to use the patas-adapted measles virus and the plaque reduction neutralization test for determinations of neutralizing antibodies against measles virus in human sera. Serum taken from a patient prior to symptoms had no antimeasles neutralizing antibodies, but one day after rashes appeared, about 90% plaque reduction by a serum dilution of 1 : 25 was observed. The titer of neutralizing antibodies continued to increase up to 6 weeks (the latest tested) after onset of measles infection in the same patient. Thus, the plaque reduction neutralization test proved to be the most sensitive method showing immune response to measles virus infection. Once the rashes appear, measles antibody is demonstrable in the blood.

To recap, the plaque technique applied to measles virus in bottle cultures under agar overlay medium is not only useful for quantitation of neutralizing antibodies in human sera, but the changing of fluid medium of Hep-2 cells when used over a period of 2–3 weeks also has been eliminated, and the incubation period was cut short to only 5 days.

Next, for more wonders of human virus plaque formation in bottle cultures, please read the following chapter (Chapter 10).

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Chapter 10

Coxsackievirus A9 variations following propagation in X-irradiated patas monkey kidney cells in culture

The wonders of patas (*Erythrocebus patas*, African red grass monkey) monkey kidney cells continued to fascinate us. PaMK cells (Chapter 1) previously demonstrated differential selectivity for enterovirus infections marking polio- and Coxsackie B viruses patas-susceptible, while Coxsackie A9 and most echoviruses are patas-resistant. The succeeding wonder of PaMK cells involves the mystery 'changing of plaque size of Coxsackie A9 virus' after serial passages in X-irradiated PaMK (IrPa) cells (Fig. 9).

At that time, Puck and Marcus (1956) reported that X-irradiation alters the sensitivity of cells in culture to virus infection. It occurred to me, 'Why not X-irradiate PaMK cells and see what effect this will have on Coxsackie A9 virus infection?' I was engulfed with fascination by the mysteries surrounding plaque variation. Believe me! Hundreds of bottle cultures were overlaid with agar medium after serial passages of

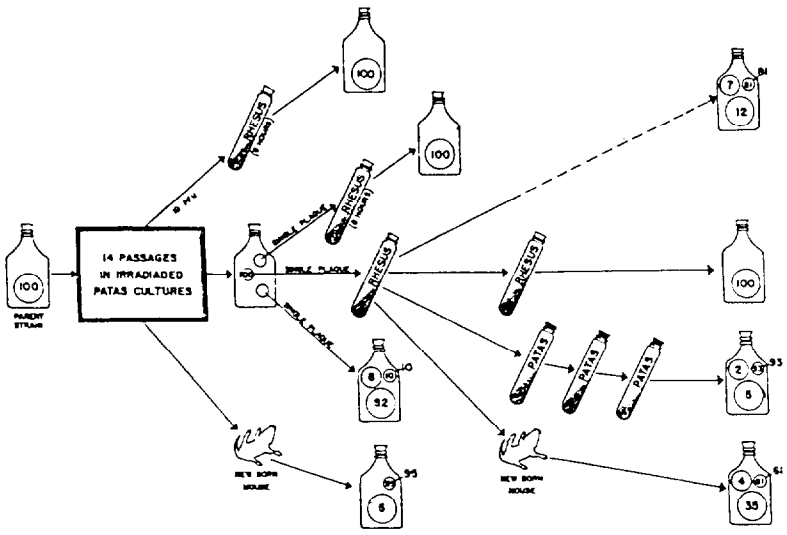

Fig. 9. Summary of plaque variation of Coxsackie A9 virus. The numbers in the small, medium, and large circles give the percentage of the small, medium, and large plaques in the populations tested (Hsiung, 1961, Yale J. Biol. Med. 33, 369).

the Coxsackie A9 virus in the irradiated PaMk cells; thousands of virus titrations, 3 repetitions of each experiment... by any standards, there was a great deal of bench work. I think now, it would be beyond anyone to do these kinds of experiments as I did, with all of the automation of the present day.

We used the non-irradiated (non-IrPa) and irradiated patas MK (IrPa) cells which were exposed to low doses of X-ray. Each passage of Coxsackie A9 virus, after replication in IrPa cells (adapted virus) was carefully examined. What do you know! On the 5th serial passage in IrPa cells, definite CPE appeared in the IrPa cells, but was absent in the non-IrPa cells serially inoculated in parallel (Hsiung, 1959). The CPE continued to intensify with higher passage levels in IrPa cells. Surprisingly, after the 14th passage in the IrPa cells, CPE also appeared in the non-IrPa cells when the adapted virus was inoculated into the non-IrPa cells.

And now, the mystical story of plaques. To begin, the parent virus only induced large plaques (greater than 10 mm in diameter) in RhMK. These large plaques were contrasted by the small (less than 5 mm in diameter) plaques of the 14th passage of the IrPa-adapted progeny. In addition, such adapted virus became less virulent in newborn mice compared to the parent virus. Both viruses, the parent and the IrPa-adapted virus strain, recovered from a single mouse brain passage when plaque tested in RhMK cells, the parent virus produced only large plaques. Interestingly, the IrPa-adapted virus produced a mixture of large and small plaques, both of which were neutralized by Coxsackie A9 antiserum. To further fascinate you, large and small plaques of IrPa-adapted virus were picked and plaque tested again in RhMK cells. Can you guess the results? The large plaque yielded large plaques only, while the small plaques yielded large, medium (5–10 mm in diameter), and mostly small plaques (less than 5 mm in diameter, see Fig. 9) (Hsiung, 1960).

What is the significance of all these plaque mysteries? It appears that the plaque size variations, taken together with the host-range, is a matter of selective pressure exerted by the two host cell systems on the virus population of Coxsackie A9 virus. IrPa cells selected the small plaque variants only, which is also less virulent for newborn mice. For as long as the adapted variant was in IrPa cell passage, the small plaque virus persisted, but when the adapted virus passaged back in non-IrPa cells it then produced a mixture of plaque sizes. Reversion from small plaque virus to large plaques of parent virus is a matter of either a single growth cycle (4–8 h) in RhMK cultured cells or only one passage in newborn mice (Hsiung, 1961).

Without the use of a modified plaque technique, i.e. plaque formation in bottle cultures, it would be impossible to define the miracle involving the adaptation of an enterovirus by serial passage in a heretofore resistant host cell system which was modified into a sensitive host cell system by X-irradiation. The plaque variation of a human virus, Coxsackievirus A9, passing through a modified host cell system presented here could not better illustrate the complexity of animal virus population selection in nature. Although this was a phenomenon as a result of laboratory manipulation, it is not far-fetched that parallel phenomena can occur in nature. It should serve as a model for understanding biological and genetic phenomena occurring with virus infections in human diseases.

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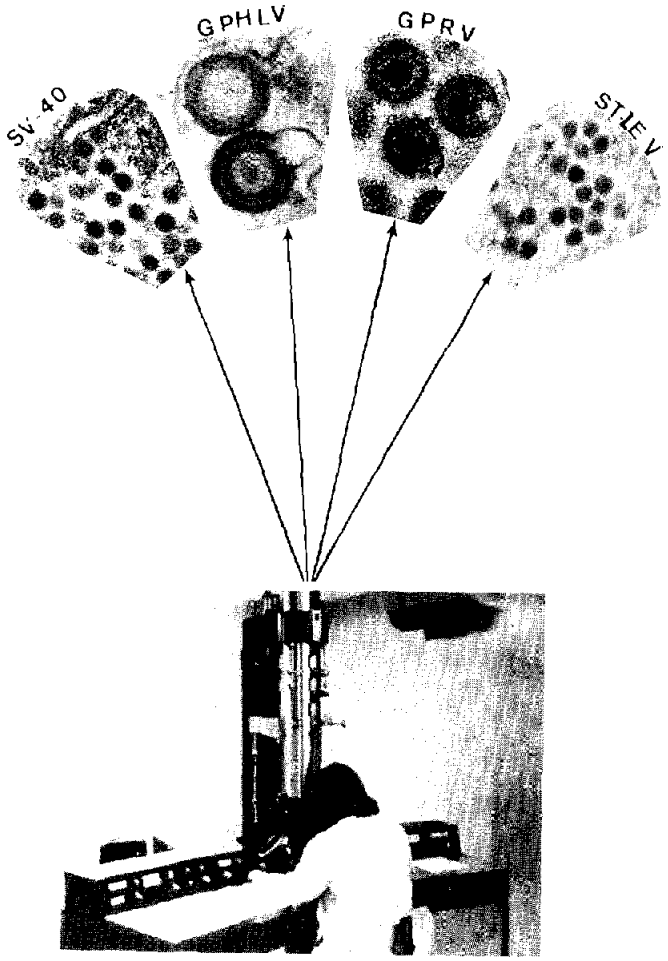
Section III

Accurate Viral Identity by Electron Microscopy



Chapter 11

Discovery of SV_{40}: the vacuolating virus of monkeys: the first polyomavirus isolated from primates

There have been reports of the presence of indigenous viruses in monkeys by several laboratories, a sequel attendant to the widespread use of cultured monkey kidney cells, in the early 1950s to 1960s. These are kidney cells derived from rhesus, cynomolgus, patas and many other monkey species. Our discovery of an unusual indigenous monkey virus is an interesting anecdote with a touch of serendipity.

Since we found that patas monkey kidney (PaMK) cells were resistant to infection by certain enteroviruses (see Chapters 1 and 10), attempts were made to alter their susceptibility to enterovirus infection. It was at this time that a report appeared indicating cultured cells, if exposed to X-irradiation, could convert their sensitivities to virus infection. Similar procedures were then applied to our cultured PaMK cells (Hsiung, 1959). A bit of history on this particular lot of PaMK cells. This patas monkey was housed for 9 months with rhesus monkeys in our monkey colony prior to sacrifice. It was in December 1957, 10 days after exposure of the cultured PaMK cells to 300 rad of X-ray, still uninoculated, but surprise (!), numerous vacuoles appeared in the cytoplasm of all the X-irradiated PaMK cells as if 'hurricane-swept'; yet the rest of the same lot of PaMK cells that were not X-irradiated remained normal up and until a holding period of 12 days.

The spontaneous degeneration was confirmed to be due to a 'vacuolating virus' designated PA-57 (patas monkey 1957) (Hsiung and Gaylord, 1961). It was noted that the vacuolating CPE induced by PA-57 virus was not transferable to rhesus monkey kidney (RhMK) cells, although it induced distinct vacuoles in PaMK cell cultures inoculated in parallel, as well as other African green monkey kidney cells. Certainly, it was a great mystery; thus a variety of biological and biochemical techniques were applied in order to characterize the new isolate from the X-radiated Patas monkey cells. These included the histocytochemical staining of virus-induced basophilic intranuclear inclusions, following cultured cells fixed in Zenker's fluid, and stained with H and E staining. In addition, the intranuclear inclusions were Feulgen-positive, indicating a DNA virus. Furthermore, filtration experiment showed that PA-57 is a small virus, less than 50 nm. After several months of extensive characterization, PA-57 virus was tentatively grouped as a small DNA virus, but there was no guideline to classify the virus into a known virus group.

It was at this time that Sweet and Hilleman (1960) reported the isolation of SV_{40} from a certain lot of poliovirus vaccine manufactured in RhMK cell cultures. However, their isolate did not induce intranuclear inclusion in infected green monkey

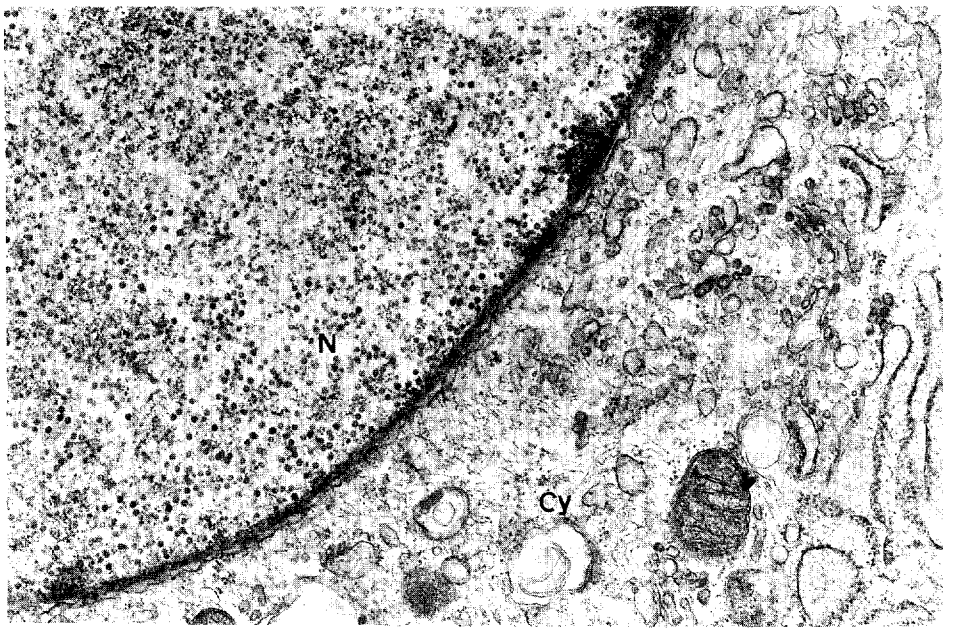


Fig. 10. SV_{40} virions scattered in the nucleus of a green monkey kidney cell.

kidney cells followed by fixing in absolute methyl alcohol. After some 6 months of searching, we found that antiserum obtained from the laboratory of Sweet and Hilleman inhibited our isolate which induced intranuclear inclusions, thus confirming that PA-57 is serologically identical to SV_{40} which showed no inclusions when infected cells were fixed with absolute methyl alcohol (Gaylord and Hsiung, 1961). In this case, the fixative fluid, Zenker's for us and absolute methyl alcohol for Sweet and Hilleman, made all the difference, the mystery! In the meantime, Dr. William Gaylord, my colleague, finally examined, by electron microscopy, PA-57 infected cells which were kept by Gaylord for several months back. To our big surprise, small virions similar to those of human wart virus were found (Gaylord and Hsiung, 1961). Thus Gaylord and Black hand-carried the EM photos of our PA-57 isolate, and drove to Washington, D.C. to visit Eddy and co-workers who reported that tumors were induced in baby hamsters inoculated with supernatant fluid from uninoculated RhMK cell cultures (Eddy et al., 1961). It became clear that rhesus monkeys apparently have a virus infection due to SV_{40}, but this never shows up until it is transferred to African monkeys, including patas monkeys. In the latter monkeys, apparent infections were accelerated by X-irradiation as in our case. It was the EM that finally identified the virus morphology into the group of viruses including human (wart) *pap*illoma, mouse *po*lyoma, and the monkey vacuolating virus; it was then proposed by Melnick as 'PAPOVA' virus group (Melnick and Papova, 1962).

Many years prior to the isolation of PA-57, 'vacuolating virus' was uncommonly encountered in PaMK cell cultures. This observation, taken with the history of co-

housing of this particular patas monkey with rhesus monkeys for 9 months prior to sacrifice, highly suggested transmission of PA-57 virus from the rhesus monkeys to the patas monkey. True enough, serological testing of all the rhesus monkeys (7 singles and 7 pools of sera) showed presence of antibodies against PA-57, while only a few patas monkeys (4/18) had SV_{40} antibodies in their sera.

In succession, a second isolate, PA-58 was obtained 7 days after PaMK cells were X-irradiated (February 1958). Then, also non-irradiated PaMK cells of this lot spontaneously degenerated 19 days after seeding. The third (1960) and fourth (1961) isolates were obtained in non-irradiated PaMK cells held 3–4 weeks after seeding.

Considering that 'vacuolating virus' also manifested in non-irradiated PaMK cells, X-ray irradiation merely accelerated the time interval for spontaneous degeneration to appear. Without X-irradiation, the spontaneous degeneration might have been missed, since spontaneous degeneration in non-irradiated PaMK cells usually appeared about 3–4 weeks after seeding; however, the usual period of holding cultures was about 2 weeks before the cells are discarded. Apparently, X-irradiation 'enhancement' can be used as a tool for screening monkey kidney cell cultures and other host systems for detection of latent viruses.

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Chapter 12
Switch between unapparent latency and apparent agents: unexpected findings in the guinea pig

The guinea pig symbol of experimentation has been widely used for scientific and biological research for decades. To my perception, it is only second to white mice in popularity as a laboratory animal. While we were embarking on full scale general studies on latent virus infections in apparently healthy laboratory animals, inbred guinea pigs of strain 2 and strain 13, and randomized bred Hartley strain were included. Strain 2, included because of its unique susceptibility to a fulminant transmissible acute lymphoblastoid leukemia, L_2C that arose spontaneously (Congdon and Lorenz, 1954), is of special interest since strains 13 and Hartley are resistant to the L_2C leukemia.

One day, Dr. L. Kaplow brought in a strain 2 guinea pig with the hope that we can find the 'leukemia virus' in this animal species since virus particles associated with guinea pig leukemia were reported (Nadel et al., 1967; Opler, 1967). As a routine, kidneys were removed aseptically from this animal. One kidney was given to a new technician, and the kidney pair was given to a research fellow for culture. The story goes that the new technician processed the kidney for cell culture on the same day the guinea pig was sacrificed, while the research fellow kept the paired kidney in the refrigerator at 4°C, and processed it the following day. Mysteriously, cell culture monolayers from the kidney prepared immediately after sacrifice, spontaneously showed areas of degeneration 9 days after seeding (Hsiung and Kaplow, 1969), while cells prepared from the second kidney which were kept at 4°C overnight remained healthy, but were found susceptible to the agent present in the degenerating cultures from the first kidney. Cellular changes were focal areas of swollen rounded cells with intranuclear inclusions characteristics of viral inclusions.

Subsequently, of the 17 strain 2 guinea pigs studied, 15 manifested spontaneous degeneration in kidney cell cultures 9–13 days after seeding. On two occasions, virus was also isolated from spleen, liver and lung tissues. Guess what? Mysteriously, homogenized tissue extracts never yielded any virus isolate. Neither strains 13 nor Hartley yielded any virus isolate whatsoever.

Hoping to quickly find virus identity, the most rapid way of presumptive identification would be by EM. Under EM examination, abundant virions with morphologic characteristics similar to the herpesvirus group were seen (Hsiung and Kaplow, 1969). Virions were free in the nuclear matrix, single membrane-bound with or without electron-dense cores. Also seen in the nucleus were closely packed double-

membrane-bound virions with or without electron dense cores enclosed within a vesicle. Particles with double membrane were occasionally seen in the cytoplasm. Serologically, no cross-reaction with the guinea pig cytomegalovirus, which is also a herpesvirus as reported earlier, could be established.

Getting back to the story — what happened to the herpesvirus in the second kidney that was kept refrigerated at 4°C overnight before cultures were made? This is how labile the latent guinea pig herpesvirus genome is: the latent viral genome cannot survive outside the intact animal too long; complete herpesvirus virions have never been found in the animal.

The story becomes more complicated when the discovery of the guinea pig retrovirus is added to this chapter. The reader is encouraged to read the following chapter (Chapter 13). As mentioned above, strain 2 guinea pigs are susceptible to transmission of L_2C/NB leukemia cells, a stable leukemic cell line. Guinea pig 'leukemia virus' particles were observed in cells and tissues of leukemic guinea pigs; however, virus particles have never been found in cells cultured in vitro. It was at this time that Lowy et al. (1971) reported that bromodeoxyuridine/iododeoxyuridine (BrdU/IrdU) can activate murine leukemia virus in cultured cells. Without further delay, BrdU was added to our cultured cells derived from the spleen of leukemic strain 2 guinea pigs with the hope of finding guinea pig 'leukemia virus'. Eureka! For the first time, cultured guinea pig cells showed virus particles after BrdU activation (Hsiung, 1972). Again, the mystery goes that these virus particles were absent in spleen cell cultures without the treatment of BrdU; while cell cultures derived from the same organs of leukemic strain 2 guinea pigs showed virus particles only after cultures were exposed to BrdU. In all instances, observations of virions were made only by EM. Most of the mature virions are in extracellular spaces; never were virions seen in the nucleus. At this point, we thought we had obtained the guinea pig leukemia virus.

Biochemically, another mystery had occurred. Repeatedly, tests showed that reverse transcriptase (RT) enzyme cannot be demonstrated with cultured guinea pig cells, although numerous virions of 'leukemia virus' were seen under EM. Guess what? At the time, Mn^{2+} was used instead of Mg^{2+}, which was necessary for the test for the guinea pig 'leukemia virus'. Thus, arguments against the identity of the virus arose.

When we first reported the above findings which represent the very first observation of the guinea pig 'leukemia virus' in cultured spleen cells, we surmised that the herpesvirus DNA originally latent in the spleen cells of guinea pigs was possibly inhibited from being transcribed if treated with BrdU, and at the same time, activated and enhanced the replication of an RNA 'leukemia virus'. With this being the case, no evidence of any herpesvirus virion in cultured cells whenever 'leukemia virus' virions were seen following BrdU induction.

For the first time, an indigenous latent guinea pig herpesvirus was identified only following cultivation; furthermore, a guinea pig 'leukemia virus' was also identified in cultured guinea pig cells in the presence of BrdU. In the latter, activation and induction by BrdU was the essential miracle, indeed, EM is a very useful tool for rapid recognition of the two unapparent latent viruses of guinea pigs following

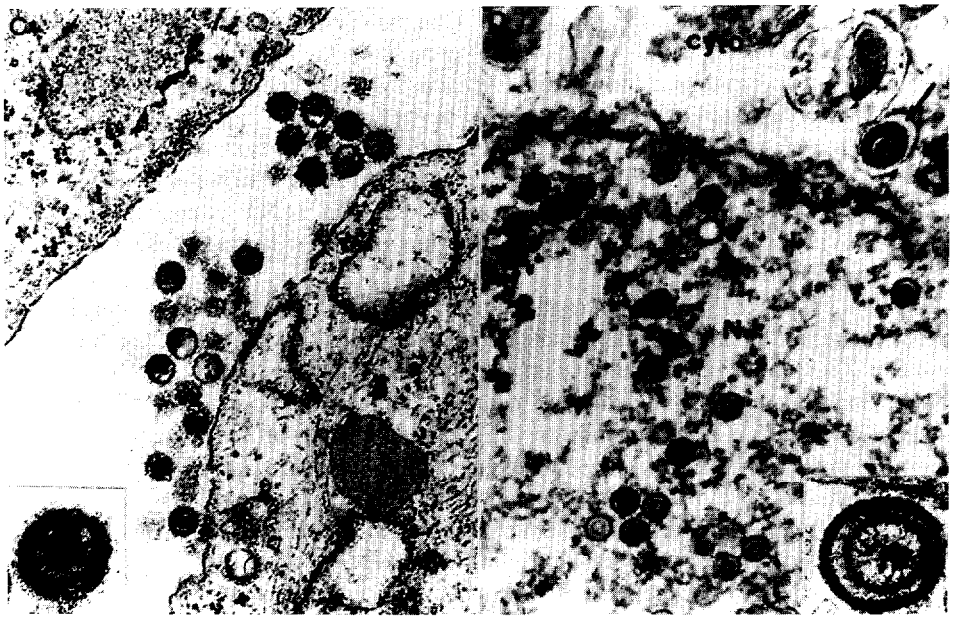

Fig. 11. Electron micrographs illustrating guinea pig retrovirus, extracellularly (left) and guinea pig herpesvirus (right) in the nucleus (N) of the same lot of guinea pig spleen cells with or without BrdU treatment, respectively. ×29,370. Inset ×100,570. Modified from In Vitro, 10 (1974) 264.

laboratory manipulations (see Fig. 11). Without EM, identification of the two agents would have been impossible.

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Chapter 13
Ever changing names of the guinea pig retrovirus

The name guinea pig retrovirus (GPRV) has been changed several times since its original observation (Table 2). In 1967, two separate groups of investigators reported the observation of virus particles in tissues and leukocytes of leukemic guinea pigs; thus named the virus as 'leukemia virus' of guinea pigs (Nadel et al., 1967; Opler, 1967). However, transmission of the disease with the partially purified virus particles from leukemic guinea pigs to normal guinea pigs was not successful (Feldman and Gross, 1970). In our laboratory, attempts were made to culture the cells obtained from leukemic guinea pigs with the hope that we can isolate the virus. It was at

Table 2
A historical account of guinea pig retrovirus

Nomenclature	Year	Significant findings	Reference
Guinea pig leukemia virus	1967–1971	Observation of viral particles in tissues and leukocytes of leukemic guinea pigs	Nadel et al. (1967) Opler (1967) Feldman and Gross (1970)
Guinea pig C-type virus	1972–1973	Discovery on activation and induction of virus particles in cultured cells derived from both leukemic and non-leukemic guinea pigs	Hsiung (1972)
		Confirmation of Hsiung's findings	Nayak and Murray (1973) Rhim et al. (1973)
Guinea pig B-type virus	1974–1977	Dispute of guinea pig virus particle morphology	Dahlberg et al. (1974)
Guinea pig oncorna-virus	1977–1980	Morphogenesis of guinea pig virus in leukemic and non-leukemic cells	Fong and Hsiung (1976)
		Biochemical analysis	Michalides et al. (1975) Nayak (1974) Schlom et al. (1977)
		Immunologic studies	Dahlberg et al. (1980)
Guinea pig retrovirus	1990–1994	Molecular biology: nucleotide sequence mechanism of activation	Doong et al. (1991) Lerner-Tung et al. (1994)

this time that two reports showed that murine C-type virus can be induced in non-producer murine cells with the addition of BrdU (Aronson et al., 1971; Lowy et al., 1971). Without further delay, similar experiments were performed in our laboratory using cultured guinea pig cells derived from both leukemic and non-leukemic guinea pigs. To our surprise, virus particles similar to those observed in leukemic guinea pig tissues were observed in cultured cells derived from both leukemic and non-leukemic guinea pigs as long as the cells were exposed to a medium containing BrdU (Hsiung, 1972). Because of the similarity between murine C-type virus and those observed in the guinea pig cells, the guinea pig 'C-type virus' was named 'C-type virus' instead of 'leukemia virus'. Immediately following our report, several laboratories confirmed our findings and the name 'C-type virus' was used (Nayak and Murray, 1973; Rhim et al., 1973). With the additional studies on the morphology of the guinea pig virus which appear to be closer to the murine B-type virus (Dahlberg et al., 1974), disputes of nomenclature as to whether 'B-' or 'C-' type should be used for the guinea pig virus were recorded in the literature. Because of the inconsistent nomenclature of the guinea pig virus, we even named it 'G-' type to represent the 'guinea pig oncornavirus' (Fong and Hsiung, 1976).

Over the years, most studies have focused on the biochemical properties of the guinea pig virus (Nayak, 1974; Michalides et al., 1975; Schlom et al., 1977). The proviral DNA sequences of the BrdU-induced 'guinea pig virus' have been found in a relatively constant amount in all guinea pig cell DNA indicating that 'guinea pig virus' is an endogenous virus system. Antigenically, the 'guinea pig virus' does not possess any group-specific antigen that is related to murine, hamster, rat, feline leukemia, RD-114, wooly monkey, or Mason–Pfizer monkey retrovirus. Since then, one study indicated that the 'guinea pig virus' may provide an evolutionary link between type B mouse mammary tumor virus and type D Mason–Pfizer monkey retrovirus (Dahlberg et al., 1980).

Interest in the studies of 'guinea pig virus' declined during the period 1980–1990. With the discovery of the successful cultivation of human retrovirus, human immunodeficiency virus, in vitro in the early 1980s, especially since it was found as the etiological agent of AIDS, there is renewed interest in reinstating studies on 'guinea pig virus' as an animal model for human retrovirus. Advances in the molecular technology led us into the studies of the nucleotide sequence of the 'guinea pig retroviruses' (Doong et al., 1991; Lerner-Tung et al., in press). Although the 'guinea pig retrovirus' is considered to be an endogenous virus of the guinea pig, quantitative analysis of the virus yield was difficult since there is no specific tool for virus detection other than electron microscopy. Even with the available, newly obtained probe, the mechanism of induction by BrdU of the 'guinea pig retrovirus' is still a mystery!

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Chapter 14
Hidden herpesviruses of equine and bovine origin as contaminants of cell cultures

The reality of indigenous viruses of healthy animals as a health hazard to handlers of cell cultures, aside from complicating viral isolation results, should always be kept in mind. Unquestionably, forewarned is forearmed. Our studies and those of many others showed that indigenous latent viruses are often recovered from a variety of animal species, especially monkeys, and laboratory animals, including guinea pigs and mice. Adding yet another indigenous latent virus to our list, herpes-like viruses have been encountered in serum samples of equine and bovine origin (see Fig. 12), commonly used as supplement for cell culture medium.

Equine species have been used exclusively for the production of antisera against a variety of human viruses. In addition, in one of our studies on latent virus infections, 9 gelding horses were included after their use for a variety of reasons, such as: source of

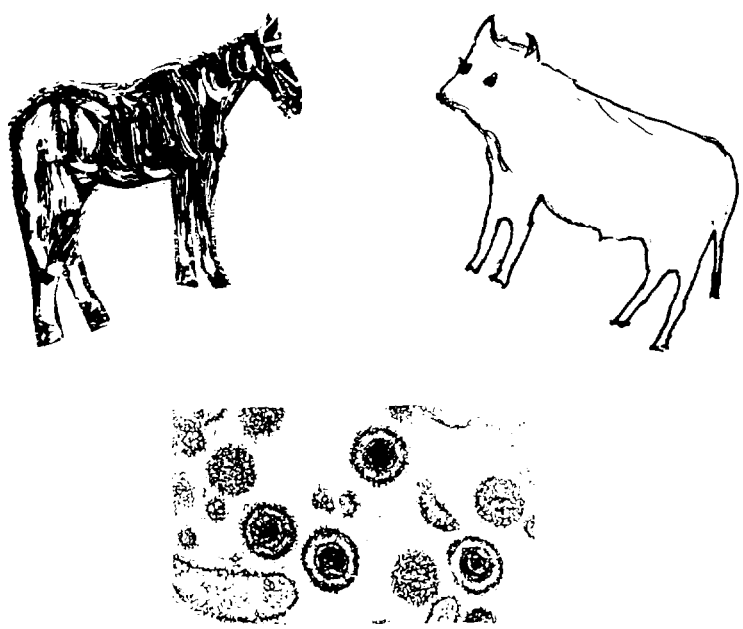

Fig. 12. Herpesvirus of equine and bovine origin.

normal serum, production of hyperimmune serum against simian adenovirus (SV_{17}), human globulin and serum, human adenovirus types 19 and 27, and diphtheria toxin. Kidney tissues obtained from 6 of the 9 horses yielded good cell growth. One of the 6 horses, 82-A, prior to sacrifice on January 10, 1967, received 7 injections of simian adenovirus (SV_{17}). Cultured kidney cells from this horse, 82-A, took 20 days to attain confluence. Subcultures were made on February 1, 10 and 20. By March 8, spontaneous CPE was noticed and became extensive by April 2 after a prolonged (50–80 days) period of cultivation, another exciting observation.

These changes appeared as small foci of rounded cells slowly progressing to extensive cellular degeneration in 3–4 weeks. Such cellular destruction can be transferred to primary kidney cell cultures derived from other horses even after 6 serial passages, but did not propagate in cell culture of other animal species' origin, suggesting an equine transmissible agent. Again with excitement, we resorted to the fastest method to identify the 82-A agent. When infected cells were examined under EM by Dr. Caroline K.Y. Fong, enveloped virions of herpes-like virus, about 106 nm in diameter, were scattered in the cytoplasmic vacuoles; whereas nucleocapsids with dense or coreless centers, were seen in the nuclei of infected cells.

Characterized further by histocytochemical stainings, 82-A virus is Feulgen-positive, hence a DNA virus is confirmed; basophilic intranuclear inclusions are seen in infected cells after H and E staining, ultrafiltration removed infectivity by millipore membranes with diameter sizes ranging from 100 to 50 nm, but passed through 300 nm, and diethyl ether completely destroyed infectivity, demonstrating the presence of lipid in the virion's structure (Hsiung et al., 1969). Serologically, the 82-A isolate was partially neutralized by antiserum against equine herpesvirus type 2, but not equine herpesvirus type 1. Antisera against human adenovirus, CMV, herpes simplex, and simian adenovirus (SV_{17}), all gave negative results. Our excitement ends declaring that 82-A virus is an equine cytomegalovirus on the grounds of morphology, together with physicochemical and biological characteristics. Had the kidney cultures not have been kept for 50–80 days, the presence of the endogenous equine CMV in cell cultures would not have been recognized.

EM, in many of our exciting quandaries in diagnostic virology, has been the harbinger of rapid successful identification of the unknown into a specific virus group, even though presumptive diagnosis, at times and often times, the most unexpected, beyond our wildest imagination.

In line with the above context, a more recent instance occurred in a lot of fetal bovine serum. Fetal bovine serum is a common nutritive supplement for cell cultures and has been for several decades. Although it has been suspected that calf serum may harbor certain viral contaminants, it is often forgotten in a clinical situation. Recently, a mystery occurred in 3 separate clinical laboratories that were using a common lot of a cell line, A549 cells (Fong et al., 1992). Similar CPE was observed in this lot of A549 cells inoculated with various sources of clinical specimens. Although CPE resembled that of herpesvirus group, immunofluorescence identification using antisera against a variety of viruses including HSV, CMV, VZV, adeno- and many others was without any success. However, when the A549 cells were fixed for EM, a herpesvirus morphology was confirmed. Without further delay, the bovine virus

was identified as infectious bovine rhinotracheitis virus, a bovine herpes virus by immunofluorescent antibody, which proved to be the contaminant in the lot of calf serum that was used to propagate the cell line. Beyond argument, the usefulness of EM in detecting and recognizing unknown agents or viral contaminants is notable. When all other methods fail, EM proves to be the most useful tool.

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Chapter 15
Identification of a viral isolate from a killer whale

Nowhere prior to the report of Buck et al. (1993) can we find a reference to show that marine mammals suffer from an arthropod-borne virus infection. At the American Type Culture Collection (ATCC), Dr. Charles Buck and co-workers have an on-going project screening virus infections of cetaceans. As part of this project, Sea World, Inc., Orlando, FL, sent pieces of postmortem brain, lung, kidney and other organs of a male killer whale (*Orcinus orca*), which was more than 25 years of age, to the Virology Laboratory at ATCC for viral cultures. Evidently, this killer whale became anorexic and lethargic in 24 h. It died 48 h after the onset of illness (September, 1990). Postmortem histopathologic examination reported a non-suppurative meningoencephalitis.

In the above laboratory, virus isolation was undertaken by inoculating supernatants of tissue suspensions of various organs including brain, lung and kidney into cell cultures, such as: primary African green monkey kidney cells, a dolphin cell line, SP-IK (ATCC CCL 78), and bovine turbinate cells (ATCC CCL 1390). On the second blind passage, SP-IK cells inoculated with either brain, lung or kidney samples showed definitive CPE. Results of this viral isolation were presented at the July 1991 American Society for Virology Annual Meeting in Fort Collins, CO. Inquisitively, I asked what the identity was of the virus isolate. Since no identification was made, I suggested that the investigators send the infected dolphin cells to our Virology Laboratory at the West Haven VA Medical Center for electron microscopy.

For several months, no infected dolphin cells arrived. The mysterious killer whale virus remained a mystery for more than 1 year. In October 1992, for the fifth time, I went to ATCC to conduct the Workshop on Diagnostic Virology. Remembering the virus isolate of the killer whale, I asked Dr. Buck to have his assistant, Grace Paulino, who actually did the virus isolation and presented the paper in Colorado, to fix some infected dolphin cells so that I could bring them back with me to the West Haven VA Virology Laboratory. As soon as I returned to West Haven, the infected dolphin cells were processed for EM examination. Nobody could have ever dreamed what the identity of the virus isolate was. Under EM, Dr. Daniel Medina, a Postdoctoral Fellow, found similar virions in all 3 samples examined. Each virion was enveloped, 40–50 nm in diameter, all were located in the cytoplasm, and budding from the plasma membrane. Based on the morphology and size, the isolate was tentatively identified as a member of the flavivirus group.

Because the virus belongs to the arthropod-borne virus group, it was suggested that the isolate be referred to Dr. Nick Karabatsos of the CDC Arbovirus Diseases

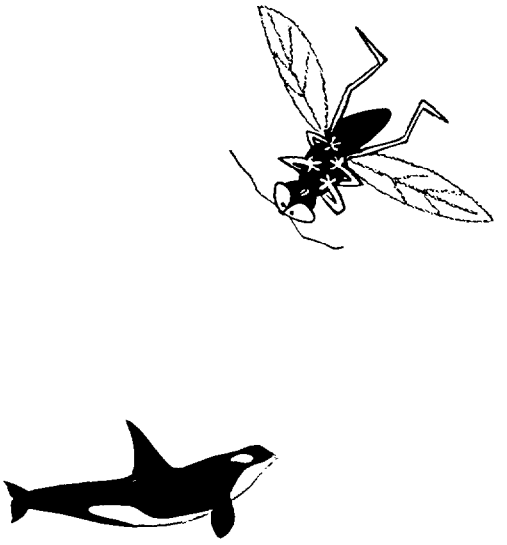

Fig. 13. When a killer whale is exposed to a mosquito carrying St. Louis encephalitis virus.

Branch of the Division of Vectorborne Infectious Disease at Fort Collins, CO, for identification. In the meantime, neutralization tests were performed in cell cultures against several viruses of the *Flaviviridae* family. Only antisera against St. Louis encephalitis virus inhibited the isolate completely in cell culture. At the Fort Collins CDC Laboratory, using immunofluorescence staining tests with monoclonal antibody, the identity of the virus isolate was confirmed as St. Louis encephalitis virus. None of the other cetaceans in the immediate vicinity of the infected killer whale came down with the same illness. Evidently, from CDC 1990 MMWR (CDC, 1990), there was a recognized outbreak of St. Louis encephalitis in Florida at the time the killer whale died. It is possible that the animal was just an incidental host (see Fig. 13), like humans who suffered severe encephalitis; most often, other animals if infected, were subclinically infected and were unrecognized.

EM in this present study, as in the previous chapters on the identification of other virus infections of monkeys, guinea pigs, horses and cows, is undoubtedly a useful tool in diagnostic virology. Not only does EM provide a rapid presumptive identification of a virus according to its size and morphology, it also simplifies the final identification process since focusing on a specific group of the unknown virus can be established. More importantly, it reveals unexpected findings, thus opening possible discoveries of novel agents of human diseases.

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Chapter 16
A case of pitfalls in diagnosis

It is often difficult to make a diagnosis based upon pathological findings alone. Life-threatening viral infections in adults are more commonly found in immunocompromised hosts. In the midst of setting up our Virology Reference Laboratory at the West Haven VA Medical Center in March 1984, a 39-year-old man with a 6-year history of chronic myelogenous leukemia was admitted to our hospital. Six months prior to his admission, he entered an accelerated phase of his disease and received a bone marrow transplant from an HLA-identical sibling at a separate hospital. His post-transplant course was complicated by both acute and chronic graft-versus-host disease. He required frequent platelet transfusions for persistent thrombocytopenia. The clinical diagnosis was graft-versus-host disease with bacterial sepsis; the patient died after a few days of hospitalization. Postmortem examination suggested disseminated cytomegalovirus infection, since intranuclear inclusions were noted in his lung, liver and small bowel (Fig. 14, top).

In the virology laboratory, samples obtained at autopsy, including lung and liver tissue, serum, and colon contents, were placed into cell cultures including rhesus monkey kidney, MRC-5, human newborn foreskin, Hep-2, A549, guinea pig embryo and rabbit kidney cells. During the first 24 h postculture inoculation, extensive cytopathic effects were observed in all cultures, including guinea pig embryo cells and rabbit kidney cultures, which were not commonly sensitive for cytomegalovirus infection. The latter observations were mysteries to us.

Before culture results were available, liver tissue obtained at autopsy was fixed and examined by electron microscopy. Within the inclusions seen on light microscopy, aggregates of viral particles 70 nm in diameter resembling adenovirus, were seen (Fig. 14, bottom). Subsequently, similar virus particles were also seen in cell cultures inoculated with the colon content and fixed for electron microscopy. The isolate was finally identified as adenovirus type 2. Human adenovirus in general, replicates only in cells of human origin. On review of the literature, it was found that certain strains of adeno-2 were capable of replication in cells from guinea pig and rabbit origin. It was another miracle (Landry et al., 1987).

In this case, the availability of electron microscopy was instrumental in establishing a definite diagnosis in both liver tissue and infected cell culture. Intranuclear inclusions noted in light microscopy are not specific for the type of virus involved; identification of viral infection often requires careful culture methods, together with virus morphology by electron microscopy and/or specific immunologic staining.

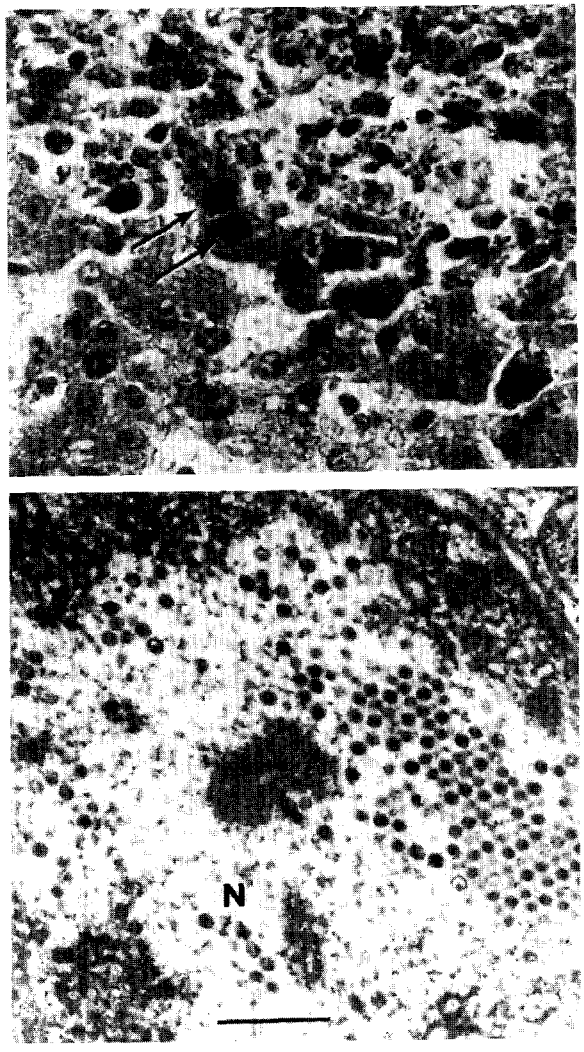

Fig. 14. Top: light microscopy of a liver section reveals two intranuclear inclusions (arrows). ×125. Bottom: electron microscopy of a hepatocyte showing adenovirus crystal arrays in the nucleus (N) bar = 500 nm. Modified from Landry et al. (1987, Am. J. Med. 83, 555).

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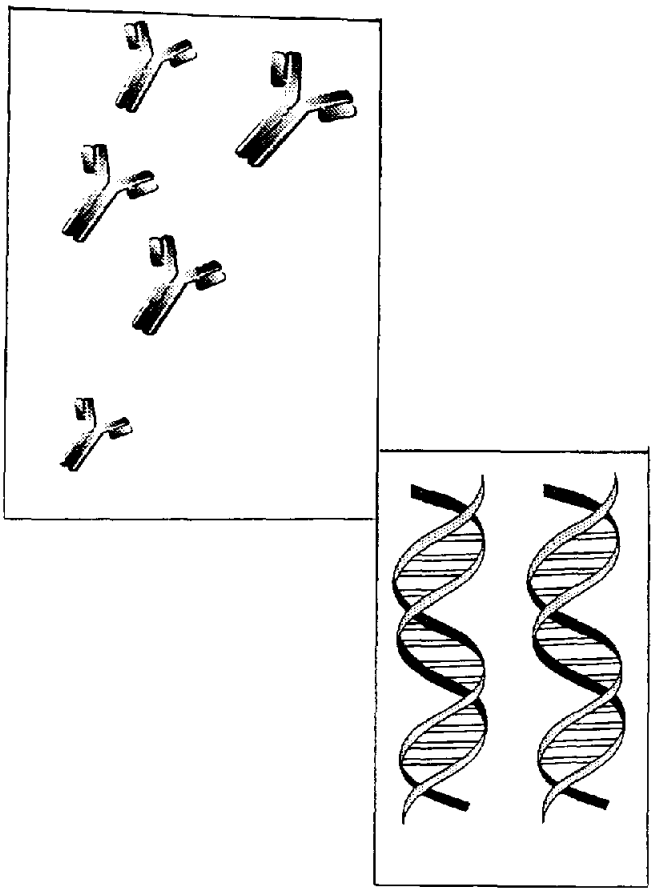
Section IV

Applications of Newly Developed Techniques for Viral Diagnosis: Pros and Cons



Chapter 17

Non-specific reaction in immunofluorescent staining test: simian vs human cytomegalovirus

What is in a name? A rose is a rose by any other name! Well, I can tell you that with viruses, notwithstanding their specific types, even their host origin matters to a great extent; hence the question 'simian or human cytomegalovirus?'

While we were undertaking a longitudinal surveillance study on cytomegalovirus (CMV) infection in primates, a strain of simian CMV, CSG, originally isolated from an African green monkey was used in the infected cells for the indirect immunofluorescent test for the CMV antibody in non-human primate serum samples. Dr. Norman Swack, the principal investigator, who usually liked to comment when things did not go well, performed the study using simian CMV-infected human diploid WI-38 cells, without any complaints. Meanwhile, Dr. Frank Michalski, who was doing a similar study using human CMV, strain Ad 169-infected human diploid cells (WI-38) for immunofluorescent tests of human serum, complained bitterly that he was not able to perform the tests due to extensive non-specific reaction. Since Frank seldom complained about anything, a big question came to mind: 'Why is it that Frank, who was working with human CMV, had so much trouble?' One day, while I was driving on highway I-95, it suddenly dawned on me... Maybe the two different sources of virus strains made the difference. When I returned to the laboratory, I suggested that Norman Swack and Frank Michalski exchange their infected cells on coverslips, that is, simian CMV against human serum, and human CMV against simian serum. It was yet another miracle! Non-specific immunofluorescence was observed only in the human CMV-infected cells regardless of the serum samples tested.

It was at that time Furukawa et al. (1975) first reported that human cytomegalovirus (HCMV) induces IgG receptors on human fibroblasts. Shortly thereafter, Keller et al. (1976) and Rahman et al. (1976) extended the findings of Furakawa et al. showing that the receptors on the infected cells are for the Fc portion of the IgG, and are produced by human CMV-infected human fibroblast cells. These IgG-Fc receptors react in both direct and indirect fluorescent-antibody (IFA) procedures, appearing as diffuse cytoplasmic or perinuclear fluorescence (Fig. 15a).

It occurred to us that the presence of these non-specific IgG-Fc receptors will complicate measurement of antibodies against human CMV in human sera when tested by IFA, which we actually found as tedious and uncertain because of intense background cytoplasmic fluorescence. Human and simian sera, either positive or negative for CMV antibodies against human CMV-infected cells caused fluorescence

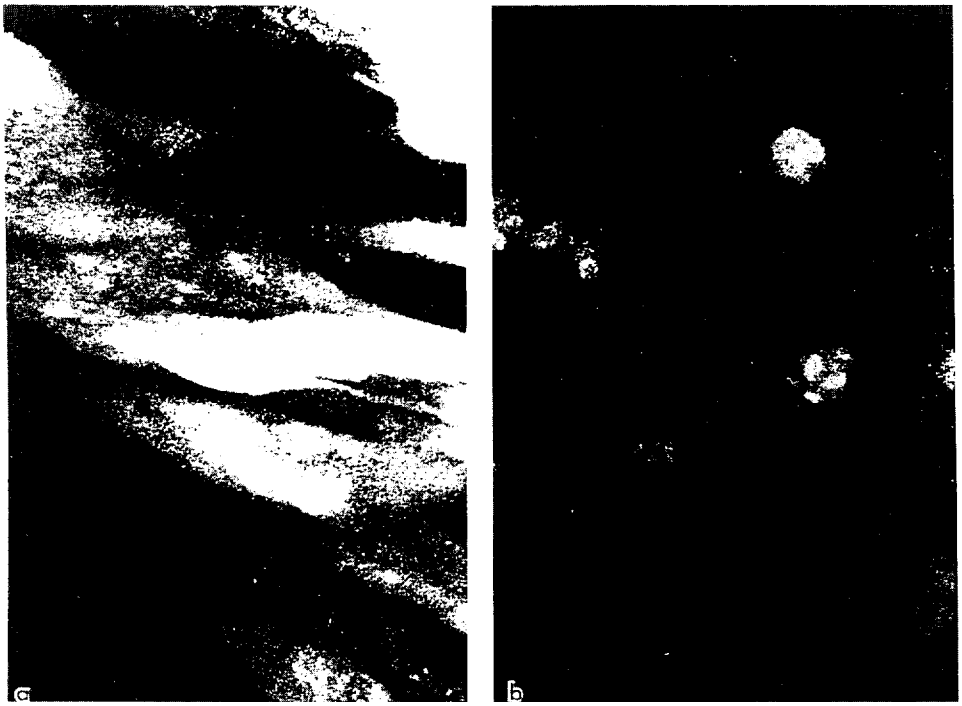


Fig. 15. Indirect immunofluorescent reactions of human diploid cell strain (WI-38) infected with CMV. Widespread fluorescence in cytoplasm and intranuclear inclusions of cells infected with human CMV strain (a). Specific fluorescence in nuclear inclusions infected with simian CMV strain (b). Modified from Swack et al. (1977, Infect. Immunol. 16, 523).

in the cytoplasm of human CMV-infected human fibroblast cells obliterating the intranuclear staining (Swack et al., 1977). Keller et al. suggested that the ability to synthesize these receptors was coded by the human CMV genome, since Vero cells, a green monkey kidney cell line, if infected with human CMV, also produce these receptors.

We inquisitively tested whether simian CMV-, instead of human CMV-infected human fibroblast cells, could be used for measuring antibodies against human CMV in human sera by IFA. The miracle unfolded beautifully; only intranuclear fluorescence was obtained with human sera containing human CMV antibodies in the simian CMV-infected cells (Fig. 15b). None of the diffused intracytoplasmic fluorescence was seen in the simian CMV-infected human fibroblast cells. Only truly positive human and simian sera stained the nucleus and not the cytoplasm of simian CMV-infected human fibroblast cells; truly negative human and simian sera did not stain either the nucleus or the cytoplasm of simian CMV-infected human fibroblast cells (Swack et al., 1977).

In this particular experience, the possible source of error due to non-specific immunofluorescence in the measurement of human CMV antibodies by IFA test has

been eliminated. Our findings unequivocally showed that CSG strain of simian CMV can, in fact, be a good substitute for human CMV-infected cells, and still be able to measure human CMV antibodies specifically in equal magnitude, indicating that human CMV antibodies in human sera cross-react with simian CMV-infected human cells as measured by IFA test. This miracle also has a 'bonus effect', i.e., simian CMV grows more rapidly than human CMV. Speed is decidedly an added advantage.

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Chapter 18
Western equine encephalitis virus mimics herpes simplex virus infection *

Immunologic techniques have been the backbone of diagnostic virology, whether it be identifying the virus directly from a clinical specimen or after culture isolation, or determination of significant increase in antibody titer in the patient's serum. The latter approach is often less useful for immediate patient management since results are obtained only after a long delay. Notwithstanding such a disadvantage, there are exceptions!

The following is a story described by an Infectious Disease Fellow, Dr. Frank J. Bia, currently a Professor of Medicine at the Yale University School of Medicine, while he was deep in the process of learning the rudiments of cell culture and isolation of viruses from clinical specimens. One day, he was contacted by Dr. George Thornton from Waterbury Hospital (Waterbury, CT), that a brain biopsy, taken from a 17-year-old boy with a case of encephalitis, was strongly suspected of being due to herpes simplex virus, based on his EEG findings. It was Frank's first attempt at diagnostic virology: isolation of the virus.

The specimen was brought from the Waterbury Hospital to the VA Hospital (West Haven, CT, about 9 miles away) by special courier, and finally arrived at midnight. Frank received the brain biopsy and processed the tissue into suspension, then cocultivated the brain tissue with a variety of the most sensitive cell cultures to herpes simplex virus (HSV) including primary rabbit kidney and guinea pig embryos, and also cell lines such as WI-38 and Hep-2. In the following days, to Frank's disappointment, no signs of virus-induced changes were noted by light microscopy in the inoculated cultures. Why did 'the isolation attempt fail'? This became Frank's first mystery in diagnostic virology. Certainly a mystery, since the spectrum of cell cultures used was known to be the most sensitive for HSV isolation and undoubtedly would support replication of HSV if any virus was present in the brain biopsy. Furthermore, electron microscopy of the brain tissue also failed to show any herpesvirus virions. The patient was in a coma and needed assisted ventilation; he was administered a 10-day regimen of intravenous vidarabine, a treatment for HSV, and his recovery was dramatic.

A second mystery became superimposed over the first one. With Frank's ambitiousness, brain biopsy suspension was also inoculated into newborn Swiss CD-1 mice.

* This chapter was contributed to by Frank Bia, M.D., Professor of Medicine, Department of Medicine, Infectious Disease Section, Yale University School of Medicine.

After 1 week of observation, the inoculated mice remained healthy. I suggested that Frank should discard those inoculated mice, but he did not. On the 13th and 14th day postinoculation, 11 out of 11 inoculated mice died. In addition, a repeat inoculation of the brain biopsy suspension into another litter of newborn mice produced similar results. Incredible but true! It was not herpes simplex virus which was the expected etiologic agent, but coronavirus, an unexpected agent, isolated from the mouse brain suspension. The latter virus was finally identified as mouse hepatitis virus by morphology, EM, and neutralization test after several weeks of laboratory investigations. What has the mouse hepatitis virus to do with the encephalitis of the patient? The miracle follows shortly!

Turning to the patient's serology, serum samples were sent to two independent laboratories: the Connecticut State Virology Laboratory in Hartford, CT and the Yale Arbovirus Research Unit, Department of Epidemiology and Public Health, Yale University School of Medicine, New Haven, CT. Both Laboratories obtained similar results: a greater than 4-fold increase in antibody titers against a California strain of Western equine encephalitis virus was obtained by complement-fixation and hemagglutination-inhibition tests. Paired sera were also sent to Centers for Disease Control in Atlanta, GA, for antibody determination of herpes simplex virus and coronaviruses of human and murine origin. All showed negative results.

It turned out that the patient had been camping in the Western United States 2 weeks prior to his illness, the likely place where he acquired his infection with a California strain of Western equine encephalitis virus (see Fig. 16). The patient's serological findings after all, however belated, have solved the mysteries surrounding the culture isolation of a virus from the brain biopsy.

Frank's important lesson (Bia et al., 1980) ... 'It is difficult to separate true viral isolates from endogenous viruses which could be present either in tissue culture or in animal systems that we used...It was a great experience, but I hope I never have to repeat it.' During the early years, monoclonal antibodies were not yet available, cell culture isolation and identification of virus from clinical specimens were tedious

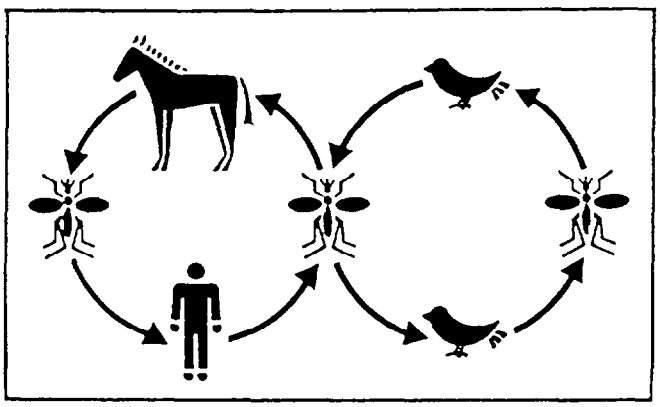

Fig. 16. Life cycle of Western equine encephalitis virus in nature.

and time-consuming processes; thus, the patient's serology was often resorted to for diagnosis when virus isolation failed! (Whitey et al., 1980).

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Chapter 19

Human immunodeficiency virus infection: a case of false-positive diagnosis by ELISA test

"Mr. Doe, your HIV-antibody screening test is positive." "What, are you kidding?" Utter disbelief runs wild in the patient innocent of any guilt associated with known practices that transmit HIV. Could it simply be a mistake; yes, a mistake of some sort? In this mystery, a patient with acute leukemia was diagnosed as HIV-positive by the ELISA test, but the truth set him free.

Although several reports of false-positives from HIV ELISA kits have been attributed to the presence of antibodies in the patients' sera to HLA (histocompatibility leukocyte antigens) class II antigens present in some HIV ELISA kits, there has been no systemic study to establish causal relation between these findings prior to our report (Yu et al., 1989), the miracle of this episode.

As thought 'luck into a vein of gold', we chanced on a male patient with leukemia receiving red cell and platelet transfusions, the star of this mystery, whose serum samples were available for HIV antibody screening tests. In the early days, antigens used in the ELISA kits were prepared from HIV-infected H9 cells, a human T-lymphocyte cell line, without realizing that this cell line expresses HLA-DR4 antigen.

It was in September 1985, the patient first manifested HIV-antibody reactivity having been prescreened for a period of 7 months showing negativity for HIV-antibody (Fig. 17). What went awry? Could it be that a mistake was committed unknowingly in this particular test? A serial follow-up, however, presented a sharp rise in HIV-antibody levels attaining its peak 3 months later (November, 1985), and then dramatically declined as fast as it rose in a span of 2 months, and returned to a negative level after 4 weeks. In general, HIV antibody levels do not decline as noted in this patient. These observations raised serious doubts in one's mind.

As such, therefore, a series of additional tests were performed. Indirect immunofluorescence assay was done with HIV-infected and non-infected H9 cells. Immunofluorescence results, somehow as the saying goes 'spilled the beans'. Positive staining was parallel along with the ELISA results, since HIV-infected H-9 cells were used in both systems. However, it was noted that the IFA staining test on the non-infected H9 cell membranes showed a pattern very distinct from that caused by HIV-antibodies.

Only after hundreds of phone calls and inquiries, was it learned that the utilization of viral antigen derived from HIV-infected CEM cells which lack human HLA antigens may give the definitive result. Focusing on the mystery, confirmatory tests

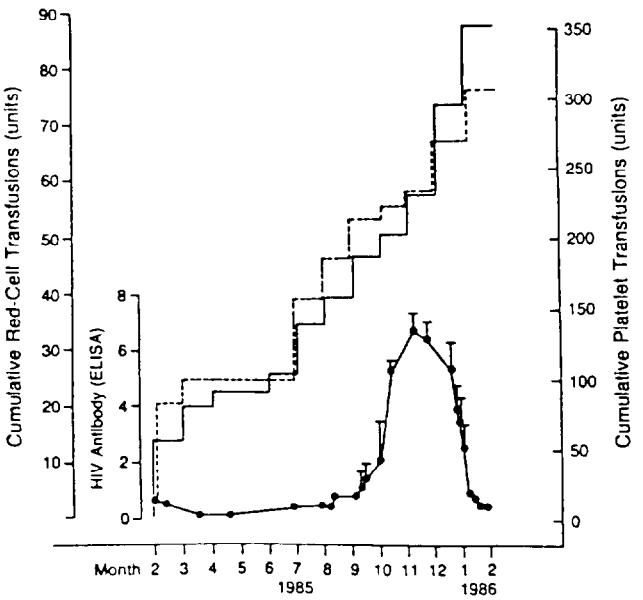

Fig. 17. Relation of serum HIV antibody levels as determined by an H9-cell-derived ELISA (closed circles) to the cumulative red-cell (solid line) and platelet (broken line) transfusions received by the patient. Reproduced from Yu et al. (1989, New Engl. J. Med. 320, 1495).

were performed by Western blot analysis and HLA-DR4 antibodies on the patient serum samples, that showed HIV-antibody positive. Truth confirmed, none of the HIV-antibody positive sera were reactive by the ELISA kit containing HIV antigen made with CEM cells which were tested by the Blood Bank of the Children's Hospital in Boston, MA, and by Western blot which was done by the Virology Laboratory at Yale University School of Medicine in New Haven, CT.

Tying both ends of the line to make a full circle, at the suggestion of Dr. J.E. Manitove, HLA-DR4 antibody titers were determined at the Milwaukee Blood Center in Milwaukee, WI. Clenching the validity of the above findings, direct evidence in fact, HLA-DR4 antibody titers were low in June 1985, rose by November (peak of the HIV-antibody), and declined to low levels in February 1986, closely in parallel with values obtained by the ELISA kit containing HIV-antigen derived from H9-cells.

The rise and fall of antibodies against HLA antigens while patients are on long-term use of blood or blood products has been well documented in cancer patients. At present the mystery, however, is serial rise and fall in HLA-DR4 antibody titers; the miracle, unequivocally established link to the HIV-antibody ELISA test reactivity using reagents carrying HLA antigens. Bear in mind that HLA sensitization by blood or blood product given by transfusions can mount an immune response that induces production of anti-HLA antibodies which are, if tested by ELISA kits containing HLA antigens, such as the H9-cell used for HIV antigen in ELISA kits,

can give strong positive reactive values. Such a reactivity can be construed as HIV-antibody positivity, when in reality, it is only a false-positive reaction. Nevertheless, it has already caused unnecessary and extreme anxiety to patients, hospital staff and associates. What agony! Beware of false-positives, and spare those concerned from anxiety and agony!

Acknowledgement

Although this study was reported as a short note in the New England Journal of Medicine in 1989, it mobilized several laboratories to prove and confirm this case. In retrospect, we are indebted to Dr. M. Hirsch of Harvard Medical School, Boston, MA, for providing both infected and non-infected H9 cells for IFA studies; to Dr. Karen Dahl of Yale University School of Medicine, New Haven, CT, for Western blot analyses; to the Blood Bank of the Children's Hospital in Boston, MA, for performing the ELISA tests using the Genetic Systems containing HIV antigen acquired from CEM cells for HIV antibody; and to the Milwaukee Blood Center in Milwaukee, WI, for testing HLA antibody titers. We are grateful to Dr. J.E. Manitove for his valuable suggestions.

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Chapter 20

Differentiating herpes simplex virus isolates by restriction endonuclease analysis: solving a 'would be' legal problem

In this modern age, people are conscious of their rights which, whenever curtailed, even to the slightest extent, would lead either to: SUE OR BE SUED! Health matters included, the 'would be' legal mystery happened in December 1979, when 3 fatalities from culture-proven herpes simplex encephalitis occurred within 3 weeks in a row in the New Haven area. One of the 3 fatalities (patient 1) was cared for by a nurse who developed herpetic lesions on her nose 12 days after patient 1 died. Alarming, isn't it? The nurse, naturally, became concerned and wanted to sue the VA Medical Center because she contracted the herpes infection from her patient, since she never had herpes infection prior to that time.

Evidence was sought; diagnostic virology employing molecular biology technique solved the 'would be' legal problem. Marie L. Landry, M.D., at that time, was on the training program for her postdoctoral training in Clinical Virology at the Virology Laboratory of the VA Medical Center in West Haven, CT. She came to the VA Hospital in West Haven, CT, all the way from Guilford, CT, on a Sunday evening to obtain a postmortem brain sample from patient 1 for viral culture. The specimen was made into 10% suspension and was used to inoculate a variety of cell cultures sensitive to the replication of herpes simplex virus, including human embryonic kidney/lung, human placental fibroblasts, primary rabbit kidney and primary guinea pig embryo cells. Advanced CPE in inoculated cultures signaled the presence of a virus isolate and herpes simplex virus was identified (Landry et al., 1983).

Subsequently, the nurse caring for patient 1 showed herpetic lesions on her nose. Landry obtained nose lesion and throat swabs from the nurse for viral cultures. Isolates from both patient 1 and the nurse were first typed by selective cell susceptibility, i.e. ability to induce plaques in chick embryo. Both virus strains failed to replicate in chicken cells and were identified as herpes simplex virus type 1. Typing results were then confirmed by restriction endonuclease analysis as follows.

Landry and colleagues analyzed 8 coded virus isolates including the 3 brain (autopsy) isolates from the 3 separate patients of the December 1979 fatalities, and the nose lesion and throat swab isolates from the nurse described above. By restriction endonucleases *Eco*RI, *Sac*I, *Bam*HI and *Hind*III, 7 of the 8 herpes simplex isolates were of distinct identity, except those of the nose lesion and throat virus isolates from the nurse that appeared identical (Fig. 18, left). Unequivocally,

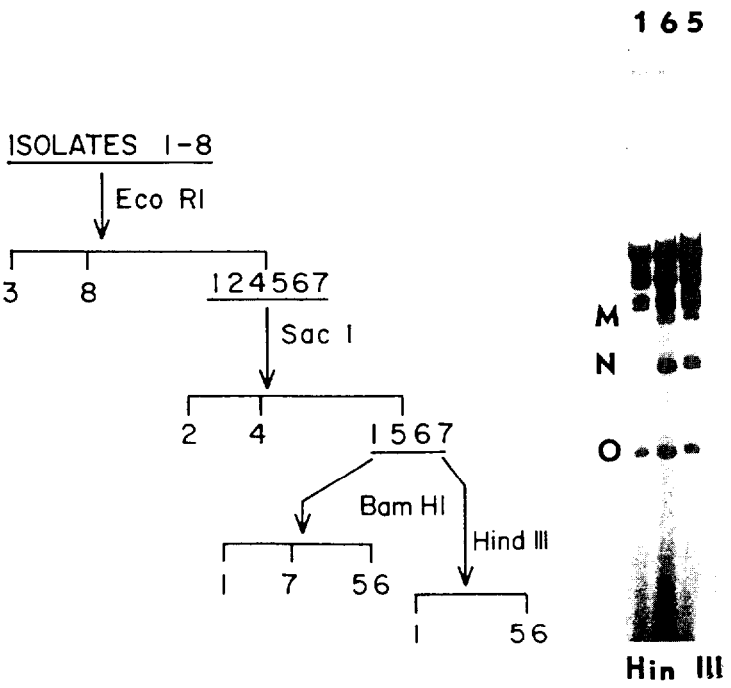

Fig. 18. Scheme of distinguishing various HSV isolates by cleavage of their DNA with endonucleases *Eco*RI, *Sac*I, *Bam*HI, and *Hind*III (left). An autoradiogram of electrophoretic pattern of the digest with endonucleases *Hind*III (right). Modified from Landry et al. (1983, Neurology 33, 831–835).

evidence obtained was lucent, the herpes simplex viruses from the nose lesion and throat of the nurse were not the same virus as that found in the brain autopsy of patient 1 (Fig. 18, right). Neither were the virus isolates from the cluster of the 3 patients (nos. 1, 2, 3) the same, nor did the two virus isolates from the nurse (nos. 5 and 6) have the same restriction endonuclease finger printing pattern as patient 1.

Restriction endonuclease cleavage site analysis has been useful in tracing nosocomial outbreaks of herpes simplex virus infections (Buchman et al., 1978) and in documenting person-to-person spread of a particular herpes simplex virus strain (Linnemann et al., 1978). The technique will validate the differences. In this 'would be' legal mystery, patient 1's herpes simplex virus was definitely different from the two isolates of the nurse who was caring for the patient. That is molecular biology in action!

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Chapter 21

The first attempt of in situ hybridization of viral DNA in paraffin-embedded tissue using non-radioisotope probes

Do you believe that things are brought together to work for the common good? Sounds biblical doesn't it? Yes! in this story of a pioneering work, it brought together, in 1980, colleagues with diverse background, to work on a common project for a specific purpose in the Virology Laboratory at the West Haven VA Medical Center. The mystery originated with David J. Brigati, M.D., a clinical pathology resident at the Department of Laboratory Medicine who came from the Sloan Kettering Hospital in New York. Doubtful of his light microscopy of histopathological readings of lung tissue sections obtained from immunosuppressed patients as to whether intracellular inclusions were actually induced by adenovirus or herpesvirus, he made inquiries as to where to find the expertise he needed to solve his dilemma. The answer he got was "Ask the help of Dr. G.D. Hsiung."

Prior to our work, the nucleic acid hybridization technique was already fashionable with the use of radioisotope-labeled probes for application in clinical laboratories. Due to the serious limitation of short shelf-life of the reagents, and to personnel because of handling isotopes and their disposal, a desirable alternative was ventured and tried. It was at this time I learned that David C. Ward, Ph.D., currently Professor of Genetics and Molecular Biophysics and Biochemistry and his associates at the Department of Human Genetics, Yale University School of Medicine, were developing a technique on the use of biotin-labeled polynucleotide probes for detection of viral DNA. In the meantime, David Myerson, M.D., Ph.D., a resident in the Pathology Department at Columbia University, Physician and Surgery College of Medicine in New York City was interested in having some experience in Clinical Virology, came to our Virology Laboratory at the VA Medical Center, West Haven, CT, as a short-term Visiting Fellow working on a project with genital herpes in the guinea pig model.

Brigati was assigned to collaborate with Caroline K.Y. Fong, Ph.D., who had had previous working-experience with adenoviruses and who had saved plenty of reagents for adenovirus work. They started the project with known adenovirus 2-infected A549 cells in cultures. David Brigati launched his quixotic search on Fong's research bench first with freshly prepared cultured cells infected with adenovirus then with fixed infected tissues. Brigati, armed with the following questions, 'Can the virus DNA in tissues already fixed in formalin and embedded in paraffin for sometime be denatured so that it can be hybridized with a biotin-labeled polynucleotide

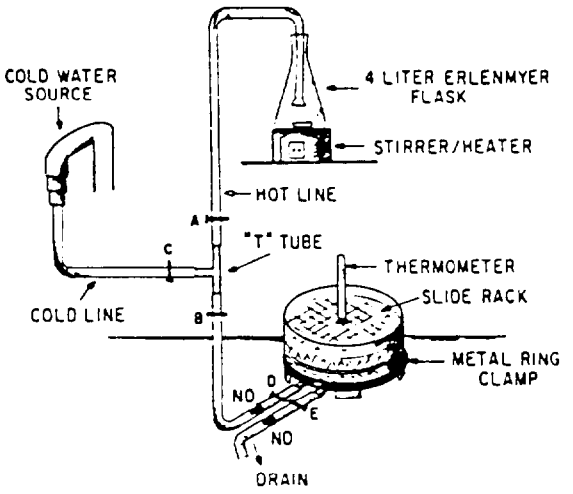

Fig. 19. Schematic diagram of the original incubation chamber for in situ hybridization where several samples can be processed simultaneously. From Brigati et al. (1983, Virology 126, 32–50).

probe?' and 'Will the hybridized DNA be immunologically detectable by either fluorescent or peroxidase staining?' The answers were 'Yes'. Viral DNA in formalin-fixed and paraffin-embedded tissues still can be denatured at 80°C to convert them into single-stranded DNA; yes, the resulting single-stranded DNA can and was hybridized with biotin-labeled polynucleotide probes; yes, the in situ hybridization was immunologically recognized either by fluorescent or peroxidase staining. Using antibiotin reagent, by any standard, all these affirmative answers spelled 'HARD WORK, DAY AND NIGHT'. The Virology Laboratory was alive till long past midnight.

You reap what you sow! From the dedication and hard work of the three 'Davids', David Brigati and David Myerson together with David Ward's cooperation (Brigati et al., 1983), it was our privilege to have been the first to report in 'Virology' on the application of biotinylated probes for adenovirus and herpes simplex virus genomes in tissue sections derived from formalin-fixed and paraffin-embedded tissues by in situ hybridization. The invention of Brigati and Myerson of a 'home-made' incubation chamber (Fig. 19) for denaturing DNA by hot water (80°C) inflow, and annealing DNA by cold water replacement deserves some comments. The chamber is large enough to accommodate 50 samples simultaneously, and the procedure required 24-h around-the-clock attention. Final findings were the visualization of herpes simplex and adenovirus DNA in formalin-fixed, paraffin-embedded autopsy tissues. Cells containing the viral genetic material were revealed by standard immunofluorescence, immunoperoxidase, or affinity cytochemical techniques that are based on the specific interaction between biotin and antibiotin IgG.

Although the sensitivity of the method developed to specifically detect viral genomes in cells and tissues following hybridization in situ with biotin-labeled

polynucleotide probes is not on par with the best of the autoradiographic detection procedures, it does offer distinct advantages. Firstly, the biotinylated polynucleotide probe can be prepared quickly, it is chemically stable and therefore usable for long periods (18 months) of time, the serious hazard of handling radioisotopes is eliminated, handling in the laboratory is safe, and feasible, wherever immunofluorescent and immunoperoxidase staining are used routinely.

Molecular biologic techniques bring wonders. If it worked with fixed tissues, Steven Spielberg's creation of Jurassic Park might still be possible... !

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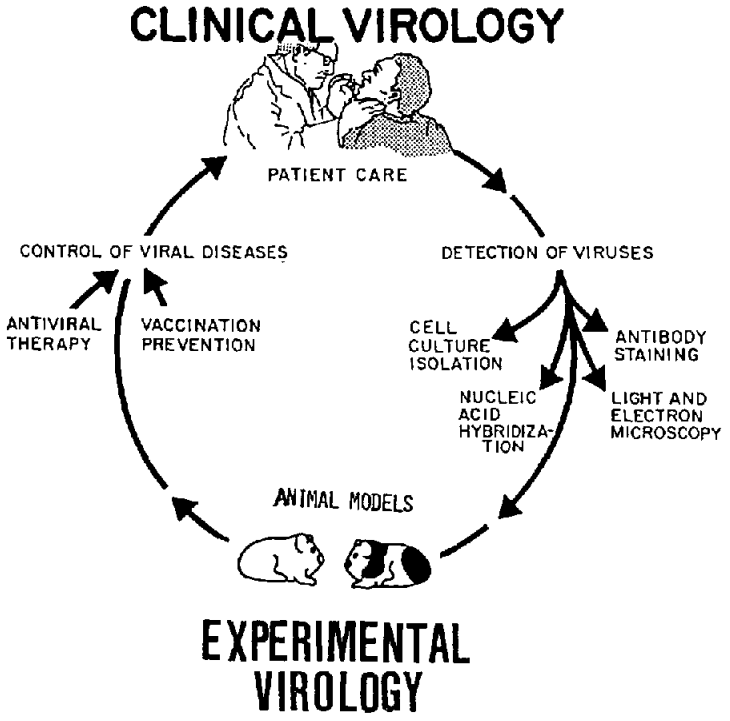
Section V

Development of Animal Models for Human Viral Diseases



Chapter 22

Finding of a herpes-like virus in guinea pig fetal tissue prompts pioneering exploration of placental transmission of cytomegalovirus infection

Originally, we were the first to report the isolation of a guinea pig herpes-like virus (GPHLV) from inbred strain 2 guinea pigs, although randomly bred Hartley guinea pigs occasionally harbor the virus (Hsiung and Kaplow, 1969). Subsequently, several other laboratories also isolated GPHLV; the virus was then named Hsiung–Kaplow herpesvirus (Bhatt et al., 1971). We surmised that since the virus was also commonly isolated from inbred guinea pigs, it must be transmitted transplacentally from mother to fetus from generation to generation.

One day Dr. Albert Jonas, DVM, at the time Director of Comparative Medicine at Yale Medical School, armed with a pregnant strain 2 guinea pig, personally delivered the animal to our laboratory door. Surely, the same virus was isolated from the lung of the fetus and several organs of the mother? Arriving on time to prove this idea, K. Lam, DVM, Ph.D., a Postdoctoral Fellow, performed a series of detailed experiments in Hartley guinea pigs. Upon the advice of the late Dr. Wallace P. Rowe, M.D. of the National Institute of Health, who instructed that strict attention be paid to decontamination of fetuses with ether baths before testing, to eliminate the chance of introducing the virus mechanically from the mother. In Lam's experiments, regardless of the stage of pregnancy at which the Hsiung–Kaplow herpesvirus was injected into Hartley guinea pigs, transplacental transmission took place. GPHLV was isolated from various tissues of fetuses from infected inoculated pregnant guinea pigs (Lam and Hsiung, 1971).

In December 1972, at Key Biscayne, FL, a conference on herpesvirus and cervical cancer was held under the auspices of the American Cancer Society. At that conference, I was invited to give a talk on our guinea pig herpesvirus, where I reported our findings that the GPHLV (Hsiung–Kaplow herpesvirus) can be transmitted transplacentally. Dr. George Klein, summarizing for that conference, considered our report 'exceptional because other herpesviruses, for example, Epstein–Barr virus (EBV) of humans, Marek's disease herpesvirus (MDV) of chickens, or Herpesvirus simirae (HVS) of monkeys are not known to be transmitted vertically'. He posed this question: 'Could this be related to relatively high permeability of the guinea pig placenta?' It was very true that the guinea pig placenta only had a single layer of trophoblasts, similar to that of humans (Enders, 1965).

Equipped with the above information that GPHLV (Hsiung–Kaplow herpesvirus) can in fact be transmitted vertically in guinea pigs, a much debated question, congenital cytomegalovirus (CMV) infection, caught our attention. R.B. Tenser, M.D., at the time, a NIH Postdoctoral Fellow, was working with us on GPCMV. He showed that CMV infection in the guinea pig is similar to that observed in humans (Tenser and Hsiung, 1976), thus strengthening the idea that GPCMV may be transmitted transplacentally in humans.

In order to prove the above idea, a systematic study on transplacental transmission of GPCMV was necessary. Robert Choi, Ph.D., a newly arrived Postdoctoral Fellow with much enthusiasm, performed experiments using randomly bred Hartley guinea pigs. Either male or female or both sexes were inoculated with GPCMV then housed in pairs, and the pregnant guinea pigs and their fetuses were examined at various periods of gestation for virus infections. Since guinea pigs have a 68- to 70-day gestation, it took a long while. Finally, Robert was very happy to examine the first 3 fetuses from the first infected female guinea pig, which was inoculated 103 days previously. However, none of the fetuses showed any CMV infection. In addition, repeated negative findings were obtained in the following 43 fetuses examined coming from 10 subsequently pregnant guinea pigs that had been inoculated for 44–150 days. Naturally, Robert was very disappointed getting all the negative findings which could not be explained at that time (a mystery), especially considering that his data were so different from our early studies on GPHLV, the Hsiung–Kaplow herpesvirus. Until one day, during our weekly conference, it suddenly dawned on me: "Robert, why don't you inoculate GPCMV into pregnant guinea pigs near term and examine their fetuses within 5–10 days postinfection?"' YES, this time, Robert performed the experiment as suggested and was excited by his findings. More than 50% of the fetuses were infected with GPCMV. Indeed, transplacental transmission of GPCMV was demonstrated only during acute primary maternal infection (Choi and Hsiung, 1978). Fetal infection occurred at different stages of gestation ranging from 28 to 60 days postinfection; however, once circulating neutralizing antibodies appeared, even if infectious virus was concurrently present in the salivary glands of the pregnant guinea pigs, infectious virus could not be demonstrated in the fetus.

Thus far, these studies with animal models have taught us many lessons on viral pathogenesis. Why are these two viruses, GPHLV, a lymphotropic herpesvirus, and GPCMV, a hemotropic herpesvirus, so different in their pathogenesis and mode of transmission?

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Chapter 23

Genital herpes in the guinea pig model: failure to demonstrate transplacental transmission, but therapy with acyclovir showed the green light

In principle, new antiviral agents must first be evaluated in animals for drug toxicity and efficacy prior to human use. It is desirable and an almost mandatory necessity to have suitable animal models for testing drugs/vaccines before human use. In retrospect, our studies on guinea pigs and several of their viral infections have, in fact, nailed down our understanding of some of the medically important human viral diseases (Bia et al., 1983; Hsiung et al., 1984). Studies on cytomegalovirus (CMV) have shown the success of transplacental transmission of the virus from mothers to their offspring which has been detailed in the preceding chapter.

It has always been questioned whether neonatal herpes encephalitis is due to the virus being transmitted vertically or by passage of the fetus through an infected birth canal. We attempted to answer this questions by drawing experimental designs from our experience on GPCMV. T.C. Chow, Ph.D., a Postdoctoral Trainee in Clinical Virology, attempted to demonstrate transplacental transmission by inoculating herpes simplex virus type 2 (HSV-2) into pregnant guinea pigs either vaginally or intracardially. Infectious HSV-2 was only recovered from nerve tissues, but not from either placenta or cord blood, or amniotic fluid from inoculated pregnant guinea pigs. HSV-2 was not isolated in any of the 53 fetuses examined. Only in one instance was a neonatal transmission of HSV-2 demonstrated from mother to newborn guinea pig via an infected birth canal (Chow and Hsiung, 1982).

Needless to say, these animal models of human viral disease are invaluable, particularly in trials of antiviral agents. Once, I attended a lecture at the New Hampshire Conference in 1981, where Daniel King, Ph.D., who at the time worked at Burroughs Wellcome Laboratories, announced that they had an effective antiviral agent, acyclovir (ACV), for the treatment of herpes simplex virus infection. From the audience, a question was raised: "Is there any side effect of the drug in pregnant women?" Daniel King's replied, "We don't know." As in a lightning flash, I thought it would be nice to test acyclovir in pregnant guinea pigs infected with CMV. To our great surprise, acyclovir was found not to have an antiviral effect on guinea pig CMV. Instead, the ACV-treated guinea pigs were all dying because of the adverse effect.

A twist unfolded. At that time, Alan Pronovost, Ph.D., a new postdoctoral trainee in Clinical Virology, was working on genital herpes model in guinea pigs. As ACV did not work on CMV-infected guinea pigs, would it work in guinea pigs with genital

herpes? As suggested, Alan tried to use ACV without any hesitation; instead of topical application for genital herpes, he injected ACV intraperitoneally in guinea pigs with genital herpes. Bingo! The genital lesions in the guinea pigs dramatically disappeared (Pronovost et al., 1982). It was an incidental finding, but with practical significance since in prior studies, ACV was found inefficient as a topical treatment for genital herpes because of the poor penetrability and absorbability of ACV. In addition, intraperitoneally ACV-treated guinea pigs showed even lower viral latency following primary infection. Furthermore, reduced incidence of paralysis of hind limbs, mortality and severity, and duration of genital herpes lesions were all evident.

Subsequently, Landry et al. (1982) extended these observations by comparing topical application vs systemic administration of ACV for treatment of genital herpes in guinea pigs. Topical application exerted some beneficial effects on guinea pig genital herpes, but was not as efficient as when administered intraperitoneally.

The significance of these findings is immeasurable as to their clinical applications. It was our good fortune to have been able to stumble on these animal models. There are many medically important human viral diseases without counterparts in animals. May the present and future generations of virologists have more luck in discovering more animal models.

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Chapter 24
An incidental finding on combined antiviral therapy

At this point in time, the top most unanswered question on AIDS is still 'WHAT IS THE CURE?' Lessons learned from the treatment of bacterial infections, notably, synergistic efficacy of paired antibiotic combinations, have become the backbone of researchers' hopes to find the much needed cure for AIDS. The 1990s may well be the decade of trials of using therapeutic anti-human immunodeficiency virus (anti-HIV) drugs in combinations with others. ERGO? Which drugs should be paired for successful combination therapy? Firm leads are nowhere for deciding the right combinations.

Several antiviral agents were made available to us during the 1980s for evaluation of their potency against CMV infection. In some of the compounds, although effective, toxicity at higher doses was a limitation. Hence, paired combinations of certain compounds with different modes of action were often considered an attractive and logical approach for reducing toxicity, and at the same time enhancing efficacy of the drug. In our first attempt to study combination of antivirals in cultured cells, we found various degrees of interaction, sometimes not knowing the reason(s), i.e. why the resultant synergism or antagonism? (Yang et al., 1990). For example, synergistic reactions were noted in vitro and later in guinea pigs against GPCMV infection using paired combinations of ganciclovir and 2′-nor-cGMP (Yang et al., 1990; Feng et al., 1992). Since the latter is a derivative of ganciclovir and has a different mode of action, a synergistic interaction was expected. However, antagonistic interactions were not expected when ganciclovir and retrovir were used in combination (Yang et al., 1990). Astounding! The potency of ganciclovir was significantly reduced when used in combination with retrovir against GPCMV in cultured cells.

Since CMV infections are often one of the most common opportunistic viral infection of AIDS patients, concomitant use of these two drugs, one against CMV (ganciclovir) and the other against human immunodeficiency virus (HIV) (retrovir) is possible. In fact, some AIDS patients may have received these two drugs concomitantly. Thus, our findings on the antagonism between ganciclovir and retrovir, though incidental and unexpected, nevertheless, has some practical implication.

To further test the antagonistic effect of retrovir on ganciclovir against CMV infection in vivo, several combinations of ganciclovir with retrovir were used to treat guinea pigs infected with GPCMV. Our in vivo data by Feng et al. (1993) confirmed our in vitro observations. It is not known, however, whether the converse interaction is true, i.e., whether ganciclovir would also antagonize retrovir potency against HIV infection.

Coincidentally, it was at this time, that Daniel Medina, Ph.D., a Postdoctoral Research Associate, was working with projects on HIV infection in our laboratory. After repeated experiments, Medina's data clearly showed that the potency of retrovir against HIV infection was also significantly reduced in cultured H9 cells in the presence of ganciclovir (Medina et al., 1992). As yet, we do not know whether such interaction occurs in humans when both drugs are concomitantly administered to AIDS patients. One report indicated that AIDS patients with CMV retinitis treated with foscarnet lived longer than those treated with ganciclovir (Studies of Ocular Complications of AIDS Research Group in collaboration with the AIDS Clinical Trials Group, 1992). The reasons for the difference are not clear. Unfortunately, no data on HIV cultures were available in that study (Studies of Ocular Complications of AIDS Research Group in collaboration with the AIDS Clinical Trials Group, 1992). It is not known whether ganciclovir used for the treatment of CMV rhinitis has played any role in these AIDS patients, resulting in the reduction of retrovir potency, although possible antiviral synergistic effect between foscarnet and retrovir may have helped those patients receiving the latter two drug combinations (Burger et al., 1993). It has been shown that synergistic toxicity of ganciclovir and retrovir, when used in combination, occurred in cultured cells and was poorly tolerated in patients because of dose-limiting hematologic toxicity, but the antagonistic antiviral activity between the two drugs has not been reported in the literature prior to our studies (Yang et al., 1990; Feng et al., 1992; Medin et al., 1992) historically, it was an incidental finding on antiviral combination therapy.

Data from our 'in vitro drug combination studies' show that in the absence of firm leads or rationale, this can be used as a guideline for selecting paired combinations of drugs for testing. As it is now, I would like to quote Jon Cohen: "How little solid ground there is for researchers to tread on as they search out the right combinations" (Cohen, 1993).

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