NEWCASTLE DISEASE VIRUS REPOSITORY

The need for collection of virus strains

Reports in the late sixties of widespread mortality in Newcastle disease vaccinated chickens of the Mideast, reports in the early seventies of hundreds of deaths among parrots being collected in Paraguay for export, and an unprecedented mortality in European pigeon lofts in the early eighties, foretold, in each instance, problems that would soon be recognized elsewhere. It is most significant that the etiologic agents of all three of these diseases were shown to be variant viruses of Newcastle disease.

Change is the nature of viruses. Within Newcastle disease virus and all other viruses is the genetic potential to exploit its primary host in new ways, to change its strategy of moving from host to host, and to expand its host range. Mutations and sometimes genetic reassortments, continually occur and take advantage of opportunities created by new interactions between host and environment. Changes in host and environment occur rapidly today favored by modern agriculture which is always housing, feeding, and managing animals in new ways as well as urban pressures that are altering opportunities for contact between domestic and wild animals.

Systematic collections for comparative study of isolates that are representative of new variants when and wherever they occur are called repositories of virus strains. Most are established by individuals with a research interest in the virus.

History of the UW repository

Carl Brandly established the repository for strains of Newcastle disease virus at the University of Wisconsin in 1949. The nucleus was a collection of strains brought from the Huntington Laboratory at Harvard University where he

worked during World War II. Beginning in 1947, isolates that were typical of the neurologic disease that became widespread in the late nineteen forties and early fifties were acquired from all over the United States and Canada. Strains were sought according to geographic origin, unusual properties, or because they had been the subject of special studies. With approval of USDA a limited number of isolates were obtained from many other areas of the world. Consequently, before 1970, the staff of the repository had received and studied isolates that produced the peracute disease that was then spreading around the world. In 1972 the repository was able to quickly identify the virus causing the epizootic of a lethal disease in California as a viscerotropic velogenic Newcastle disease virus. Over a hundred highly virulent isolates were characterized before that exotic virus was eradicated from California. As a system of quarantine stations for exotic birds was placed into operation to avoid further importation of Newcastle disease virus, it became necessary to trace the illicit movement of diseased birds for forensic reasons. Development of the oligonucleotide fingerprinting method made it possible to show the identity of isolates believed to be associated ~~or~~ or their unrelatedness, and to provide scientific evidence for prosecution of smugglers in court.

Similarily, at Weybridge, in England, Dennis Alexander working with isolates from the British repository of Newcastle disease virus strains developed a monoclonal antibody screening method that established the relatedness of Newcastle disease isolates from an epizootic in pigeons and later showed that this infection had spread to chickens. The development of these molecular virological methods made virus collections essential and the epidemiology of the virus more readily interpretable.

Earlier, F.R. Beaudette at Rutgers University screened a large collection of isolates for strains of reduced virulence for chickens. Three of his

candidates for use as live virus vaccines became commercial vaccines and two, LaSota and B1, are ~~to~~ the primary strains used worldwide today.

The extent of the differences among strains

Wide differences exist among strains of Newcastle disease virus in their relationship to hosts and in their physical properties. The deviation is extreme for many of the properties that were formerly used to separate viruses into species such as, host range, tropism, physical stability, and antigenic character. On the other hand, the morphology of the virions of all strains appears to be the same under the electron microscope with the exception of rare particles that are twice as large and probably contain two nucleic acid strands. While nucleic acid of the virus has recently been sequenced, studies of the homology of strains have not yet been done. However, oligonucleotide fingerprinting of perhaps 50 representative strains reveal considerable differences and one would suspect that there are a number of variable sequences where differences would be found among strains.

Virulence for chickens

Best known and documented of strain differences is virulence for chickens, a property that was once used as a justification for separating a mild form and a severe form and calling one pneumoencephalitis and the other pseudo-fowl pest or Newcastle disease under the assumption that they were caused by different agents.

Virulence is measured by the severity of the disease induced, the time required to express the disease and the quantity of virus required to produce a disease response. While the process is dependent upon the age of the host and its genetics, immature individuals being more sensitive, and to a minor extent upon environmental conditions, the primary determinant is strain of

virus. Approximately 10 virions of a highly virulent strain such as 1083 is all that is required to induce an acute lethal infection by either natural routes (i.e., oronasal) or by injection (i.e., intracerebral). In contrast, a billion-fold greater dose (10^9 chicken embryo ID^{50} or 10^9 plaque forming units) of an avirulent strain such as B1 or Ulster, irrespective of route that they are given, including intracerebral injection, fail to produce disease or death in chickens. Many strains are intermediate. When given by the oronasal route, a lethal dose, depending on the strain, may contain 1,000, 10,000, 100,000, or a million CE ID_{50}. Other strains, like Roakin, produce mild or inapparent infections at any of these doses by the oronasal route, but a dose of only 10 to 100 CE ID_{50} if given by the intracerebral route is rapidly lethal. By any criterion the spectrum of interaction between virus and host exhibited by strains of Newcastle disease is impressive.

The other two measurements of virulence (a) the time required to express disease and (b) nature of the disease response also depend upon strain. The incubation period, the time between exposure and development of first detectable sign which usually is respiratory distress is short, approximately 48 hours even for mild strains and is highly dose dependent. Prostration and diarrhea, which are signs of severe disease, appear between 72 and 96 hours. Neurologic signs; tremor, distortion, and paralysis, usually develop later, between 4 and 10 days. Unlike the incubation period, the time between infection and death is highly strain dependent. Chickens exposed to peracute infections die on the fourth or fifth day, with death of all exposed birds closely clustered in time. Strains that are a little less virulent kill chickens between the sixth and tenth day. The death pattern of less virulent strains is spread over a longer time period, and some chickens may survive with or without sequelea.

The tropism of strains that induce inapparent or mild infections is limited to either the respiratory or digestive tract. Even when clinical signs are inapparent in such infections, histologic changes can be seen in the infected epithelial cells. These strains can be recovered from tract openings, by use of nasal, tracheal, or cloacal swabs, and specific antibodies can be detected in the peripheral blood. Attempts to isolate virus from other tissues, except in rare instances, are unsuccessful.

In acute and peracute infections, virus can be found in almost every tissue of the body and lesions are widespread and characterized by extensive vascular damage. While there are difference among them, all virulent strains produce high titers of virus in many tissues and do it quite rapidly. Strains that produce neurologic disease have a longer course and fail to induce hemorrhagic lesions in the gut. Only birds that have not yet developed vaccinal immunity or whose immunity is wanning survive long enough to develop neurologic signs following exposure to the peracute enteric strains.

Virulence for day old chicks, chicken embryos, and cell cultures

Immature chickens are very susceptible to Newcastle disease virus. Many strains that produce mild or inapparent disease in adult birds produce severe and even fatal disease in birds during their first few weeks of life. Only the lentogenic strains produce inapparent infection in chickens less than a week of age and therefore can be safely used as vaccines. Chickens that are a day old will survive intracerebral injection with lentogenic strains without developing signs of disease. However, wet chicks less than 24 hours old exposed to the same virus by this route usually die and day old chickens inoculated in the yolk stalk with lentogenic strains may also succumb to infection.

Ten day old chicken embryos are a very sensitive and useful laboratory hosts for Newcastle disease. Following intraallantoic inoculation, all strains produce hemagglutinins in the allantoic chamber that become detectable within about 24 hours. Strains that are virulent for chickens kill embryos in 48 to 72 hours and those that are avirulent for chickens kill embryos between 96 and 120 hours. As this death pattern is both dose and strain dependent, standardization of the dose is required to permit comparison of strains. Some of the mildest strains may fail to kill all embryos. However, all strains kill embryos rapidly (48 hrs) if inoculated into the yolk sac of ten day old embryos.

While all strains of Newcastle disease virus infect chicken embryo fibroblasts, lentogenic strains require the magnesium ion and DEAE to produce visible plaques. In the presence of these additives, the virulence of Newcastle disease virus for chickens can be predicted from the size of the plaque. Minute plaques, < 0.1 ml, if cloned, produced inapparent infections. Large plaques, > 4 ml, if cloned, produce severe and fatal infections. Intermediate plaque sizes produce disease of intermediate severity. Some Newcastle disease strains in addition to the clear plaques just described (in which the virus-killed cells do not stain) also produce red plaques (in which the infected cells are deeply stained). Large red plaques like large clear plaques contain chicken lethal virus, and small red plaques like their clear counterparts contain virus that produces mild disease.

Virus isolated from mild and inapparent infections produce only indistinguishable small clear plaques. Virus isolated from severly diseased birds invariably contains two or more kinds of plaques distinguishable by their type and size. Some isolates have as many as 6 easily distinguishable kinds of plaques that can be cloned and shown to have distinct properties. Only clear plaques are isolated from mild and velogenic neurotropic strains

while red and clear plaques have been obtained from the velogenic viscerotropic strains.

Virulence for other species

Virulence of Newcastle disease for chickens is a poor predictor of its virulence for other species of birds. Many of the strains that cause an acute viscerotropic disease in chickens produce inapparent infection in such passerines as the pitta and flowerpecker, mild or inapparent infections in ducks and geese and severe or fatal disease in psittacines. The relationships are quite complex as several clones of a viscerotropic velogenic strain that were incapable of inducing clinical disease in chickens caused a fatal disease in canaries. An isolate from pigeon that regularly produced a fatal infection in racing pigeons generally produced only a mild infection when inoculated into chickens.

Newcastle disease occasionally infects people. The severity of the response, primarily a conjunctivitis, has no relation to the virulence of the virus for chickens. The virus also induces disease in several laboratory mammals. The best studied is the mouse which when exposed intranasally may develop pneumonia and when exposed intracerebrally can succumb to a fatal encephalitis. Both responses which are characterized as viral toxicity rather than infectious, are strain specific and the response appears to be unrelated to virulence for chickens.

Antigencity

A great deal of emphasis has been given to the antigenic simularity of Newcastle disease virus isolates. It is true that inapparent and mild infections caused by lentogenic strains induce antibody that will protect chickens against the severe and peracute disease caused by viscerotropic

velogenic strains. However, significant differences have been shown between the ability of some strains to induce polyclonal antibody that is equally effective in all heterologous and homologous combinations. One way neutralization differences have been observed that are as great as 10,000 fold when the reciprocal cross showed little or no difference.

Monoclonal antibodies that combine with particular reactive sites have been prepared for HN, M, F, and NP proteins. Seven distinct sites have been found for some proteins and there may be many more. It appears that certain reactive sites are subject to considerable variation and others are conserved. Seven clones derived from a single virulent strain were found to be all different by one set of 17 monoclonal antibodies and to have the only clone that was different by another set of 7 monoclonal antibodies.

Hemagglutination

The hemagglutinin neuraminidase glycoprotein and the fusion protein of Newcastle disease virus have enzymatic activities that are expressed by their action on cell surfaces. Tests based on these reactions in vitro often reveal differences between strains.

While all strains of Newcastle disease virus agglutinate chicken erythrocytes and probably all avian and reptilian erythrocytes as well, they differ sharply in their ability to agglutinate various species of mammalian erythrocytes. For example, horse erythrocytes are agglutinated by B1 strain but not by LaSota strain. Optimal conditions for agglutination of erythrocytes differ among strains. B1 usually fails to agglutinate chicken erythrocytes at 8°C but agglutinates them at 26 C and 37C. Most other strains have the same agglutinating activity at all three temperatures.

Hemagglutination is a time dependent process in which readings in the pattern test can not be made earlier than 25 minutes (controls are not yet

negative) or after 45 to 60 minutes when the positive sheet begans to break up and indistinguishable "button" deposits form in both positive and negative wells. The disaggregation of cells is called elution and the rate at which elution occurs, as readily determined by resuspension, is strain dependent.

The hemagglutination activity of the virus is destroyed by heat and by chemicals. Below 50°C inactivation is slow and above 60°C it proceeds very rapidly. A temperature of 56°C gives the best resolution of strain differences. The hemagglutinating activity of some strains is destroyed in 5 minutes and that of other strains still remains after 4 hours of heating. Heat resistance of virus infectivity is somewhat related to that of the hemagglutinin. Strains whose hemagglutinating activity is destroyed in five minutes usually retain their infectivity for about thirty minutes. In contrast, strains whose hemagglutinin was not destroyed until after several hours of heating at 56°C becomes non infectious in about ninety minutes. The disassociation of these activities for Newcastle disease is unlike the close linkage of these two activities observed for influenza virus. The thermostability pattern of clones derived from a strain may be similar or different. It is possible to select thermoresistant subpopulations for some but not all thermosensitive strains. However when selected the thermoresistant line has the same virulence as the strain from which it was derived and thermosensitivity and thermoresistance occur in wild type strains irrespective of their virulence for chickens.

A further catalog of strain differences, binding by lectins, sensitivty to acid and bases, ability to form syncitia, attachment to brain cells--the list could go on--would only provide additional evidence that there are genetic differences among strains. It is more important that attention be given to the processes and the problems associated with making collection of strains.

Acquisition of a repository of virus strains.

Rationale of collection. While a collection of isolates that is representative of the biological potential of a virus may be acquired by acceptance of spontaneous offerings, active solicitation of unique isolates is more likely to meet the goal. There are five considerations: 1) A collection should contain isolates from all geographical regions as diversity is more often found among cultures obtained at a distance from each other. The relationship is not direct as unusually diversity has been found within small regions. For example, vesicular stomatitis virus and venezuelan encephalitis virus have more genetically distinguishable forms in Central America than in the much larger regions of North or South America. 2) Isolates from species representative of the virus's host range should be acquired. Newcastle disease virus from pigeons and parrots were clearly distinguishable from virus routinely recovered from chickens. While ND virus recovered from some abberent hosts such as the calf and man have often been of laboratory origin, occasional isolates such as the Kemerovo virus reputedly isolated from a fatal human infection in Russia have unique properties that merit preservation irrespective of questions about their origin. 3) Virus that is associated with particular epizootics or with enzootic foci should be preserved and have made it possible to trace the probable origin. 4) Sequential collections year after year have shown evolutionary change. The last isolates from the California epizootic were less virulent than the early ones. 5) Any strain should be acquired that has been the subject of a significant study, so that its the nucleic acid sequence is known or its hemagglutinin or neuraminidase characterized or its pathogenesis in a host well described or that has been used to produce a vaccine or reagent. The development of almost any information about a strain increases its value to other investigators. Use of representative strains rather than a single laboratory culture enables an investigator to determine whether an observation is trivial or substantive.

Operation of a virus repository

On acquisition, the history of the isolate must be documented, its identity and purity established, stock cultures prepared using procedures that protect against contamination and genetic change, and aliquotes packaged for long-term preservation in storage.

Information that identifies a culture is as important as the culture and should include more than a name or designation. Strain history sheets help donors provide comparative data. While no form will be appropriate for every occasion, a simple list of questions is enough in some instances and in other instances a questionnaire as elaborate as the strain registration form used by the American Arbovirus Committee will serve the needs.

Information is needed on at least 6 topics. 1. Host species from which the isolate was recovered and the nature of the specimen taken. 2. Location where the host had been maintained or captured and the date that the sample was obtained. 3. Nature of the disease observed in the host and its associates. 4. Procedure used for isolation, particularly the culture system and significant observations about the procedure. 5. Name and address of the laboratory (and individual) who made the isolation. 6. Passage, preservation, and storage history.

Donors of derived cultures--clones and culture adapted lines, etc. should enlarge the last entry to include passage history before derivation, the process of derivation and subsequent passage. The correspondence and history sheets should be retained in a documentation file, along with data on strain behaviour and citations in the literature.

The curator of the repository is now faced with two tasks.
1) verification of the identify, purity, and activity of the culture and
2) production of a stock of an unaltered culture.

The first is straightforward. Neutralization of the virus in a sensitive host system by use of a reference antisera verifies the antigenic identity of

the virus. Bacterial contamination is not uncommon and if present, can be eliminated in most instances by antibiotic treatment of the supernatant of the centrifuged preparation. Occasionally a contaminating virus is revealed when the known virus is neutralized. With information from the behavior of the unknown contaminant in selected laboratory host systems, it is often possible to identify it by use of a small set of reference antisera and eliminate it.

When subjected to scrutiny, contaminating virus has been detected frequently enough in cultures that are exchanged by investigators to make it evident that routine procedures to guard against contamination are not in place in many laboratories. It follows that data exists in the published literature that has been compromised by the undetected presence of a contaminating virus.

Any stock that will be used to generate additional virus stock in contrast to of virus that will be destroyed in the course of use should be handled within a restricted protocol. When being prepared and used as an inoculum and when being harvested from a host system, the stock virus should be the only infective agent in the working area. When it is centrifuged, lyophilized, or similarly manipulated the virus should be handled alone and the equipment being used should have been previously sterilized. Outside of a host system, the virus must be kept in sealed and clearly identified containers. The use of cryptic codes that are not easily interpreted by all members of the laboratory staff can lead to misidentification or destruction of the culture because of fear of incorrect identification. These restrictions are essentially the same as those required by most governments of any producer of vaccines and reagents.

In studies of laboratory safety, almost every deviation from the recommended security practices has been identified as a possible cause of contamination (1) mislabeling and inadequate identification of cultures, (2) failure to decontaminant inoculating or harvesting equipment or work areas and

special equipment, (3) improper segregation of cultures in storage. Before the biologics industry was adequately regulated, commercial fowl pox vaccine was found to be contaminated with Newcastle disease virus, probably from the work area, and Newcastle disease vaccine was found to be contaminated with avian leukosis virus and mycoplasma, (probably originating from the host system). Detection of Newcastle disease virus in 12 cultures derived from blood of people suffering from a febrile illness was mystifying until it was learned that the research team making the isolations, while never occupying the laboratory on the same days, shared their laboratory with another team who were searching for influenza virus in birds. The first team used suckling mice for isolation and the influenza team used embryonating eggs. The Newcastle disease isolates obtained by the two teams were indistinguishable. Contamination probably occurred in an inadequately decontaminated centrifuge or lyophilizer.

The contaminating virus may be a strain of the same virus. Isolation of avirulent cultures of Newcastle disease virus from chickens exhibiting severe respiratory-enteric disease may be possible but it is more probable that the avirulent isolates were laboratory contaminants.

The selective effects of laboratory culture systems on wild-type virus which often contains several subpopulation should be a major concern. Burnet and his colleagues observed changes in the behavior of influenza virus after less than three passages in embryonating chickens eggs that they called the O-d transformation. Some wild type strains of Newcastle disease virus have undergone similar changes in transmissibility and virulence after as few as one or two passages. Most changes in Newcastle disease virus on passage are usually associated with pronounced shifts in their plaque populations. Change from a mixed population of large and small plaques to one of small plaques has been associated with decreased virulence and change to a uniform population of large plaques is associated with unchanged or increased virulence. Change in

properties may be unassociated with a shift in plaque morphology, but the apparently identified plaque population has become resistant to an inhibitor or to physical condition of storage.

Selection is determined not only by the host system and methods of storage. Procedural difference such as multiplicity of infection (inoculating dose) and time of harvesting virus have been demonstrated to be selective. Beaudette observed that a technician who harvested the first embryos that died obtained a virus line that was more virulent for chickens than did a technician who always harvested the last embryos that died. Techniques such as these that unintentionally select virus of different properties may yield variant populations after a few or many passages. Deliberately selective methods, such as the careful removal and culture of an isolated plaque result in rapid change. A relatively pure population can be obtained this way on the first passage and within a few passages one can secure a clone of virions that are uniform in genetic composition.