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CHAPTER 35

METHODS FOR POTENTIAL APPLICATION TO RODENT VIRUS ISOLATION AND IDENTIFICATION

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A number of virus detection methods have been mentioned at this conference. Those routinely used in the rodent virus diagnostic laboratory have not yet approached the level of sophistication generally achieved in studies of these agents as experimental models. The purpose of this paper is to present an overview of methods commonly used for isolation and identification of viruses infecting laboratory rodents. In most cases, systematic comparisons of the specificity and sensitivity of these methods have not yet been made. However, some of the conclusions drawn about specific methods will be based upon reports from human and veterinary diagnostic laboratories. New techniques with potential for application to rodent viruses will be discussed, as will the necessity for rapid diagnostic methods.

Viral and Mycoplasmal Infections
of Laboratory Rodents

I. GENERAL CONSIDERATIONS

Because of the expense and labor-intensive nature of most virus detection methods, a differential diagnosis should be considered before laboratory tests are performed. A differential diagnosis may be based on clinical presentation. Frequently, however, rodent virus infections are subclinical, and diagnostic requests stem from experimental results which do not conform to earlier experiences. Serologic tests, histologic examination and a thorough review of husbandry and environmental factors may suggest explanations. If there is reason to suspect an infectious etiology and if a sound differential diagnosis can be established, the appropriate samples may be submitted to the diagnostic laboratory.

Consultation with the diagnostic laboratory will prove helpful to the investigator at this point. Special instructions related to the collection, storage or shipping of specimens may be discussed. For example, if virus recovery is the method of choice, specimens should be collected aseptically and maintained in sterile containers, but should be kept on wet ice for as short a period of time as possible. Specimens collected during the acute phase of disease are more likely to yield positive results than are those collected after an outbreak or during convalescence. If the diagnostic laboratory is remote from the submitting facility, specimens packed on dry ice for transport should be within tape-sealed vials to prevent exposure to carbon dioxide.

II. DETECTION OF INFECTIOUS VIRIONS

In a recent review of methods for rapid diagnosis of human virus infections, virus isolation was termed the "gold standard" to which all other methods should be compared (1). Virus isolation results in identification of the etiologic agent as well as its preservation for further studies and experimental reproduction of the infection. An additional advantage of virus isolation methods is their ability to detect the unexpected. Assays which use specific probes are likely to detect only the agents against which the probes are made, whereas virus isolation methods may detect an agent which was not suspected. There are circum-

stances in which animal use is mandatory for virus isolation. However, in view of the ease of transmission of many rodent viruses, alternatives to animal inoculation may be preferred for virus detection.

For many rodent viruses, we prefer in vitro isolation in cell cultures susceptible to the agent(s) of interest. In vitro methods are less expensive than animal inoculation and results are often obtained more quickly. As an example, isolation of reovirus type 3 from a transplantable neoplasm of mice required more than two weeks after inoculation of newborn mice; however, isolation and identification were accomplished within seven days of inoculation of susceptible mouse L cells. Further, isolation of lymphocytic choriomeningitis virus (LCMV) by intracranial inoculation of adult mice required seven to ten days, whereas isolation and identification were accomplished in 48 hours using susceptible cell cultures. LCMV, which usually does not induce any detectable cytopathic effect (CPE), was identified by indirect immunofluorescence using a reference immune reagent. A variety of manipulations may be used to increase the probability of infection of cultured cells. One example is the use of diethylaminoethyl (DEAE) - dextran in the absorption medium to enhance uptake of virus into cells. This has been used successfully to increase titers of MHV-3 in DBT cells (2). In the same study, plaque size was increased by the inclusion of trypsin in the overlay medium.

After inoculation of cell cultures, identification of the infecting virus is a critical step since many rodent viruses, especially those recovered from animal tissues, do not induce morphologic changes in cultured cells. A battery of reagents suitable for use in immunofluoresence or enzyme immunoassays is essential. These reagents should not react with uninfected or mock-infected cells.

Other methods available to detect the replication of animal viruses in cell culture include detection of a hemagglutinin (HA) or complement-fixing (CF) antigen in the supernates of infected cultures. The former method obviously requires that the agent possess an HA antigen, while the latter method is problematic because of the stringent molecular configuration requirements of the CF test (3). The detection of HA activity has been recommended as a diagnostic tool for human adenoviruses (4) and for canine parvovirus infection (5). In the latter case, tests using chloroform-treated fecal specimens were reported to be rapid and specific. The hemagglutination inhibition (HAI) test, used for confirmation of specific HA activity, also discriminates CPV from other autonomous parvoviruses, such as feline panleukopenia virus, mink enteritis virus and

minute virus of canines (5). The HAI test similarly discriminates among rodent parvoviruses (6). For the mouse parvovirus, minute virus of mice (Crawford strain), Parker et al.(7) reported that induction of CPE in rat embryo cells was a very sensitive method which could be supported by detection of HA or CF antigen or by immunofluorescence. Immunofluorescence staining of primary mouse embryo cultures has been used to detect antigens of K virus which did not induce CPE (8). Carthew (9) used horseradish peroxidase-labelled antibody to demonstrate MHV antigen in L929 cells exposed for less than 24 hours to clinical material from naturally infected mice. In our experience, MHV isolates generally induce CPE within 24 to 48 hours, and we have never detected antigen in a culture which was CPE-negative.

Hemadsorption (HAd) and hemadsorption inhibition (HAdI) tests have been applied to Sendai virus (10); however, the tests are very time-consuming and must be read microscopically with several replicate determinations for each sample.

A novel approach to the quantification of infectious virus has been the application of enzyme immunoassay (EIA) technology to monolayer cultures of infected cells (11). Using a peroxidase conjugate, countable foci of herpes simplex virus-infected fetal lung cells were visualized as "plumes" of reaction product which diffused into the supernate. The assay was equivalent in sensitivity to the fluorescent focus assay and could be interpreted at 17 hours post-infection, well before the onset of cytopathic effect. The method was also successfuly applied to the detection of infectious centers of mumps and measles viruses in Vero cells. We have modified this method for the detection and quantification of rodent viruses in cultured cells (11a). The assay is equivalent in sensitivity to the fluorescent focus test which we have applied to several rodent viruses (12, and unpublished data), and its specificity can be readily demonstrated by neutralization of infectivity. The technique is easily applied to large numbers of specimens, requires small volumes of reagents and does not induce the eye fatigue experienced after prolonged viewing with a fluorescence microscope.

Cell culture methods for virus isolation cannot readily be applied to all rodent viruses, since some grow poorly or not at all in continuous cell lines. A systematic survey of commonly used cell lines may yield sensitive host systems for these agents. Trypsin treatment is known to enhance rotavirus replication in cultured cells (13,14). Other manipulations, including the use of hypertonic medium and the incorporation of cortisol, retinoic acid and vitamin B12

into the culture medium (15), have been used to increase rotavirus yields. Preparation of cells from a primary origin, as is currently required for *in vitro* cultivation of rat coronaviruses, is time-consuming and expensive. Virus isolates with restricted cell or tissue tropisms may also be difficult to recover. An example is our recent finding of a strictly enterotropic strain of MHV which did not replicate in the normally permissive NCTC 1469, 17 Cl 1 or L cell lines, but which did grow in CMT-93 cells derived from a murine rectal carcinoma. This virus did not replicate in normally permissive cell lines even after passage in CMT-93 cells.

III. DETECTION OF VIRAL COMPONENTS

A. Antigen

1. Immunocytochemistry

The probability of virus recovery may be reduced by a variety of factors. If isolation attempts are made after the appearance of neutralizing antibody, the proportion of apparently infectious virus decreases rapidly. In addition, viruses produce many more noninfectious particles and often more structural proteins than infectious virions. Theoretically, then, methods which detect viral components could be more sensitive than those which detect infectious virus. Viral proteins, including structural proteins and non-structual or virus-specific enzyme proteins, can be detected in tissue sections using fluorochromes or enzyme immunohistochemistry. These methods have been useful for diagnosis and have aided in the detection and identification of previously unrecognized agents (e.g., ref. 16); however, they do not allow physical isolation of the agent.

Immunofluorescence staining of frozen sections has long been used to localize viral antigens in tissues. More recently, methods have been developed which allow immunochemical staining of tissue sections which have been processed as for histological examination (17). In the human diagnostic setting, these methods have been generally less sensitive than isolation techniques (1). Isolation methods can sample an entire tissue, whereas antigen detection methods usually sample only a small section of that tissue. Hall and Ward (18) have recently reported differential

preservation of antigenicity of Sendai virus in mouse lung tissue by several fixatives, so it would seem wise to compare several procedures prior to undertaking antigen detection studies in clinical material.

In human diagnostic laboratories, antigen detection has been used to identify viral products in various body secretions. The sensitivity of immunofluorescence for respiratory syncytial virus with nasopharyngeal washings was 95% compared to virus culture methods (19), and the specificity of the reactions was reported to be 86.5%. We have attempted to identify rotavirus antigen in fecal smears from infected mice by immunofluorescent staining and have found that interpretation is quite difficult, presumably due to nonspecific interactions of antibody-containing serum with multiple antigens present in feces.

Among the disadvantages of immunofluorescence are 1) the need for a fluoresecence microscope and high-quality reagents, 2) autofluorescence of some tissues, and 3) the requirement for interpretive experience, because determinations of endpoints are relatively subjective. Immunoperoxidase, on the other hand, requires only a light microscope and permits antigen-specific staining on a background of standard histologic staining. Endogenous peroxidase activity may interfere with interpretation, but methods are now available to minimize such activity (20). Neither technique has the potential for automation, so processing of large numbers of samples at one time is not possible.

Electron microscopy (EM) may also be used to detect virions and viral antigens in infected tissues. Ultrastructural studies are expensive and time-consuming, and unembellished EM is relatively insensitive. We have recently developed an immunoelectron microscopic technique for identifying rabies virus antigens and sites of virus synthesis in the peripheral and central nervous systems of infected mice (21). This procedure, which takes advantage of colloidal gold-conjugated Staphylococcus aureus protein A binding to the IgG fraction of immunoglobulin, has as its main advantage over EM the ability to identify virus-associated structures which would otherwise be morphologically unidentifiable. Low-temperature embedding of tissue was crucial to the preservation of antigenic reactivity of rabies virus proteins. While the technique is aesthetically pleasing and extremely useful in ultrastructural studies of viral pathogenesis, it is not recommended as a routine diagnostic procedure due to the cost and time required for its performance. EM has, however, revealed the presence of viral and mycoplasmal contaminants of transplantable tumors which were otherwise unidentifiable in our laboratory (22).

B. Radioimmunoassay (RIA)

Clearly, sensitive, less expensive and time-consuming methods are needed for antigen detection. RIA is very sensitive and can be performed rapidly with large numbers of samples using automated equipment. However, the hazards and expense associated with the use and disposal of gamma-emitting isotopes suggest that enzymne immunoassays may be a preferable alternative.

C. Enzyme Immunoassay (EIA)

EIA offers the advantages mentioned above for RIA; however, some of the enzymes are unstable and some of the substrates are carcinogens (26,27). The current expense of automated washing equipment and spectrophotometers may be prohibitive for small laboratories with limited resources. Nevertheless, since EIA's have now been extensively tested in human diagnostic laboratories, some discussion of the prospects and problems associated with them may be useful.

Several variations of the EIA are now available. The literature suggests a wide array of possible methods, enzymes and substrates. Most antigen detection EIA's involve the adsorption of antigen to antibody "captured" on a solid phase, usually wells of microtiter plates or plastic or metal beads. A recent variation involves the use of nitrocellulose filters for nonspecific capture and immobilization of antigen (23). Because the filters are held within a vacuum manifold conforming to the 96-well microtiter format, addition of reagents and washing may be done quickly and efficiently. In addition, acid-glycine treatment may be used for the elution of native antibody which might otherwise interfere with the detection of antigen and/or raise the level of background signal in a traditional solid-phase assay.

The two most commonly applied enzymes for EIA are horseradish peroxidase and alkaline phosphatase. The former is a plant product and is readily available at reasonable prices. Its dark reaction product facilitates visual interpretation. However, peroxidase loses its activity after contamination with microorganisms, and antibacterial agents such as methanol or sodium azide also interfere with its activity (24,25). In addition, some peroxidase substrates are carcinogens (26,27), and the available fluorescent substrates are quite unstable (24,28). In contrast, alkaline phosphatase is very stable and resistant to the

action of bacteriostats (28). Its substrates have not been shown to be biohazardous. Since it is purified from an animal source, its supply is more limited and it is more expensive than peroxidase.

Other enzymes less frequently used include betagalactosidase and glucose oxidase. These have the advantage of being absent from body fluids at pH 7, and may gain applicability in the clinical setting by virtue of this property.

EIA's have generally been found to be less sensitive than isolation for detection of human virus infections. For detection of respiratory syncytial virus, EIA was comparable in sensitivity to immunofluorescence; however, only 78% of culture-positive specimens were detected (29). For Coxsackie A and B viruses, about 60% of culture-positive samples were detected by EIA (30,31). The usefulness of EIA was, thus, limited by the necessity to perform isolations on EIA-negative specimens. Several manipulations have been tried to increase the sensitivity of the assays. The indirect method, while increasing the number of incubations and washes, is more sensitive than the direct technique (32,33). An assay using the ability of C1q to bind to antigen-antibody complexes also increases sensitivity (34,35), but its usefulness is limited when endogenous circulating immune complexes are present. The competitive assay, in which antibody either mixed with specimen or unbound binds to antigen on the solid phase, overcomes this problem; however, antibody present in clinical specimens causes false positive reactions. This is a particular problem if the clinical specimen is serum or cerebrospinal fluid. A complement-magnified EIA has been reported which is 10- to 100-fold more sensitive than the standard EIA for human rotavirus detection (36); unfortunately, nonspecific reactions are also magnified by this method.

There is sentiment that antigen detection EIA's have reached the limit of their potential sensitivity (1). The reasons for this are as follows. The requirement for extensive washing to remove unbound reagents results in dissociation of specific antigen-antibody complexes. This elution will occur unless the antibody used is of very high affinity and avidity. This requirement suggests that monoclonal antibodies will be of limited value in EIA's since they are generally of very low affinity. The use of pooled hybridoma products may help to overcome this difficulty. Secondly, the interpretation of EIA's is based on the relative strengths of the specific and nonspecific (or background) signals. Nonspecific binding of enzyme to the solid phase or to a clinical specimen results in the

conversion of substrate to product. Modifications which amplify the specific signal generally increase the background signal. Test sensitivity is also highy dependent on the variability among replicates. This variation is usually fairly high for clinical samples, which are not purified and are not at all homogeneous. On the other hand, use of a substrate which yields a precipitable product has the potential to distinguish between products of specifically and nonspecifically bound enzyme (1). In addition, this method was reported to detect a single herpes simplex virus-infected cell.

Additional considerations applicable to EIA's, at least for detection of human agents, include the binding of a rheumatoid-like factor in serum and stool to the Fc portion of immunoglobulin, the accessibility of internal antigens and the frequent problem of antigen-antibody complexing in clinical specimens. The problem of Fc binding is apparently dependent on the age of the specimen donor and on the species source of the reference immunoglobulin (24,37). Antigen accessibility has been improved by treatment of specimens containing respiratory syncytial virus or influenza virus with N-acetylcysteine or of specimens containing adenovirus with sodium dodecyl sulfate (29,36,38). Such treatments can also denature viral antigens, and enveloped viruses present a special problem in this regard.

Clinical specimens often contain endogenous antibody, and a number of treatments have been used to eliminate such antibody. The use of high affinity antibody to coat the solid phase can enhance successful competition for antigen that is eluted from antigen-antibody complexes. Physical methods, such as heat, extremes of pH, and treatment with denaturing agents or enzymes have been used with polysaccharide antigens (39,40). Care must be taken to reverse these conditions prior to addition to the solid phase, because antibody can also be denatured by these treatments. Reversal may be accomplished by cooling, dialysis or treatment with anti-proteolytic agents, respectively. Sodium thiocyanate has also been used to dissociate antigen-antibody complexes; however, the reversal of the treatment results in reaggregation of the complexes. Methods such as ultracentrifugation or gel exclusion chromatography are effective, but are inefficient in the human situation requiring rapid diagnosis. Treatment with mild reducing agents such as cysteine-HCl or glutathione have no effect on antigenicity, but do denature IgG and IgM, and should be removed by dialysis (41). Clearly, better methods for the dissociation of immune complexes in clinical specimens are needed.

Because of the difficulties associated with culturing rotaviruses, a number of techniques have been investigated for diagnosis of infection. For detection of antigen in calf feces, an EIA was found to be more sensitive than EM, immunofluorescence, CF or immunoelectrophoresis (42). In addition, EIA was simple to perform and was suitable for processing large batches of clinical specimens. A commercial EIA was tested for its ability to detect rotavirus antigen in specimens from pigs, horses, cattle, dogs, sheep and exotic ruminants and gave accurate results within 24 hours of receipt of specimens (43). In another study of human rotavirus diagnosis, EIA was compared to EM and solid phase aggregation of coupled erythrocytes (SPACE), an assay similar in concept to EIA except that antibody- coated erythrocytes replace the enzyme-substrate reaction as indicator (44). SPACE was less sensitive than EIA, and EIA was equivalent in sensitivity to EM for rotavirus detection. However, the EIA was quicker, less labor-intensive and less expensive than EM. Treatment of rotavirus antigen with chaotropic agents such as NaSCN or guanidine-HCl have been reported to increase the sensitivity of EIA by three- to five-fold due to enhanced immobilization of antigen on the solid phase (45).

An area of EIA methodology which has not received adequate attention is direct measurement of viral enzymes. Such an approach requires the absence of the enzymes from host cells or fluids or the ability to distinguish between enzymes of viral and host origin. The neuraminidases of influenza and parainfluenza viruses have been detected with EIA's using the fluorescent substrate 4-methylumbelliferone (46,47). Bacteria also contain associated nueraminidases, and their presence in clinical specimens can hinder the interpretation of assay results. The thymidine kinase of herpes simplex viruses has a differential activity with various substrates compared to kinases of mammalian origin. An assay based on direct detection of this enzyme detected two virus-infected cells in one sample (48,49). Detection of the DNA polymerase of hepatitis B virus was associated with false positive reactions due to the presence of mammalian and bacterial polymerases with similar reactivity (50). An assay to detect RNA-dependent RNA polymerase has not been evaluated, but has promise since host cells are incapable of synthesizing RNA directly from an RNA template.

Using poly-L-lysine to bind antigen to the solid phase and pooled immune serum as a marker, an EIA has been developed to detect a broad spectrum of human enteric viruses (51). These included Coxsackie A-9, B-3, and B-5, echovirus 6, polio type 1 and reovirus type 1. The assay did not

detect two porcine enteroviruses and was rapid and inexpensive. Although it was less sensitive than virus isolation, this EIA was thought to have potential for detecting some important, noncultivatable human enteric pathogens (e.g., Norwalk agent, hepatitis A). However, stool specimens present special problems for the development of EIA's because they contain multiple extraneous antigens.

B. Viral Genomes

Nucleic acid hybridization has been applied in the diagnostic setting to a number of human viruses, including Epstein-Barr virus, hepatitis B virus, herpes simplex virus (HSV), human rotaviruses, adenovirus and cytomegalovirus (52-57). Genome probes with high or low specificity can be produced. The former will detect a specific virus, whereas the latter may detect any virus in a complex. Complementary nucleic acid strains bind to each other with high avidity, so that hybridization assays are quite sensitive. As few as four HSV-infected cells or 10,000 infectious units of virus may be detected by this method (1).

Hybridization assays do have some drawbacks. Their sensitivity at present is roughly equivalent to that of EIA's, but they require both sophisticated equipment and isotopes which are expensive and difficult to dispose of. One solution has been the conversion from isotopic probes to the use of biotinylated ones which can be detected colorimetrically (58,59). These probes may be used with nucleic acid which has been immobilized on filters or with tissues in situ. They have a long shelf life (at least one year) and result in low levels of nonspecific binding. Their use has reduced the problems of isotope disposal and the time formerly required for the development of autoradiographs.

In a study comparing hybridization to culture for detecting influenza virus infection in nonhuman primates, viral RNA was detected for several days after the animals became culture-negative (1), suggesting that hybridization is worthy of further development as a diagnostic tool. This result is consistent with the concept that production of neutralizing antibody interferes with the ability to detect infectious virus.

In man, hybridization findings must be interpreted with caution. In studies of chronic neurologic syndromes (multiple sclerosis, amyotrophic lateral sclerosis and postpoliomyelitis weakness), hybridization in situ has been demonstrated with material from control patients (60,61).

Waksman and Reynolds (61) termed such findings "molecular archeology" and emphasized the difficulties presented in the diagnostic setting by the detection of viral genomes of agents to which patients were exposed years prior to the onset of their chronic disorders.

Hybridization has not yet been applied to rodent viruses in a clinical setting, although hybridization to frozen whole body sections of suckling mice has been reported for polyoma virus (62). We are using biotinylated probes in studies of viral pathogenesis, and we have also applied a nucleic acid detection procedure to the double-stranded RNA virus, EDIM (63). Genes of double-stranded (ds) RNA viruses can be separated on the basis of molecular weight on polyacrylamide gels or, after glyoxalation, on agarose gels (64,65). Electrophoresis of ds RNA's is one of the techniques that serves as a basis for the relatively new discipline of "molecular epidemiology." The usefulness of the method for murine rotavirus detection lies in the fact that EDIM, which grows poorly in all cell cultures so far tested in our laboratory, can be detected directly in fecal material which is extracted, labelled at the 3' ends of the RNA molecule with 32P-pCp, and electrophoresed (63). Several samples can be processed simultaneously, allowing differentiation of isolates by comparison of RNA migration patterns.

IV. IMMUNE RESPONSE AS A DIAGNOSTIC TOOL

Serologic testing of paired acute and convalescent serum samples, with diagnostic rises in antibody titers between the first and second blood collections, has traditionally been used in studies of viral epidemiology. This approach surely taxes the resources of large decentralized laboratory animal facilities. However, the immune response has been used extensively as a criterion to detect rodent virus infections. Detection of an anamnestic antibody response after challenge has been called a method of choice for the diagnosis of MHV (66) and ectromelia virus (67) infections. As a routine diagnostic procedure, this technique seems to pose unnecessary risks to an animal facility.

One application of immune responsiveness in the diagnosis of rodent virus infections has been the antibody production test. There is renewed interest in this test as a diagnositc method because serologic tests with greatly enhanced sensitivity are available and because monoclonal

antibodies and genetically engineered products are now being produced for eventual clinical trials in humans. Many of these reagents are being made in rodents or rodent cells that must be tested for contamination with infectious agents (68), including conventional rodent viruses. A distinct advantage of the antibody production test over direct isolation procedures is the fact that animals produce antibody to both live and inactivated virus; however, the immune response to inactivated virus or protein subunits is usually inferior to that following inoculation of whole, replicating virus (3). Therefore, the usefulness of this procedure for detecting nonreplicating virus or viral subunits should be examined carefully. Caution must be exercised in the interpretation of antibody production tests, the results of which are only as reliable as the serologic tests performed.

Parker et al (7) reported that the mouse antibody production (MAP) test was more sensitive than *in vitro* induction of CPE for identification of field strains of MVM; however, other *in vitro* detection methods, such as immunofluorescent staining of inoculated cells, were not compared. Rowe and co-workers (69) compared development of HAI antibody in the MAP test to a CPE induction test in primary mouse embryo cultures and to tumor production in suckling hamsters for titration and detection of polyoma virus. The tissue culture and MAP tests were comparable in sensitivity, reproducibility and time required for completion. At limiting dilutions, CPE was apparent after 14 to 32 days, while HAI antibody was usually present at 21 days. Determining tumor production was problematic due to difficulties with reproducibility. The antibody production test has not been systematically compared to newer methods of virus detection. In view of the potential human applications of rodent products, the sensitivities of antibody production tests relative to EIA's, immunofluorescence and other methods should be compared in order to determine the most reliable safety test.

V. RAPID DIAGNOSIS: IS IT IMPORTANT IN THE RODENT VIRUS DIAGNOSTIC LABORATORY?

Rapid diagnosis is important in the human virus diagnostic laboratory for a number of compelling reasons. Chief among these is the fact that there are now antiviral drugs which can significantly alleviate the course of some virus infections. Since a single syndrome may be caused by

several different agents, only one of which may respond to a certain antiviral, it is important to distinguish among the possible etiologies. This is particularly important if the drug has toxic side-effects or is extremely expensive. Rapid diagnosis may also contribute to the prompt initiation of epidemiologic control measures, such as vaccination campaigns. Although patient management will probably never be the norm in a rodent colony, prevention and control can be of utmost importance, especially for zoonotic agents such as lymphocytic choriomeningitis virus or Hantaan and related viruses. In addition, several contributors to this symposium have emphasized the impact of intercurrent infection with any of several rodent viruses on basic research. Thus, both zoonotic potential and the impact of infection on research objectives should be considered in decisions regarding the need for rapid methods.

VI. METHODS APPLICABLE TO THE STUDY OF RODENT VIRUS EPIZOOTIOLOGY

Several methods exist to study the movement of viruses within and among rodent colonies and to distinguish between vaccine virus-induced infection and wild type infection. Perhaps the most frequently used method to differentiate viruses for epidemiologic studies is the neutralization test (NT). The kinetic or timed NT is a variation which allows discrimination among very closely related viruses which cannot be distinguished by the traditional NT. By assaying the degree of neutralization at frequent, early intervals after the mixture of antibody and virus, rates of neutralization can be calculated prior to reaction equilibrium. One issue which should be addressed here is the form of the antibody that should be used for successful detection of differences between or among virus strains. Hyperimmune sera or ascitic fluids are often used, because the titers of such reagents are significantly higher than those of single-shot immune sera. However, hyperimmunization can result in the induction of more broadly cross-reactive antibody which is less likely to discriminate among closely related virus strains (70). As an example, Childs et al.(71) recently published the results of cross-neutralizing antibody assays using six reference strains of mouse hepatitis virus (MHV) and hyperimmune ascitic fluids prepared against five of the strains. Their data suggested that some of the strains are

so closely related as to be indistinguishable by neutralization. They conclude that kinetic neutralization tests are "more precise... for determining antigenic relationships between murine coronaviruses." In contrast, we have found that the same reference MHV strains are easily distinguished from one another by the traditional NT (72), providing that single-shot immune sera are used as the source of antibody.

The ability of ectromelia virus to agglutinate mouse erythrocytes has been reported as a reliable method to differentiate between infection with this agent and infection or vaccination with other poxviruses (73).

Oligonucleotide fingerprinting has been applied as an epidemiologic tool to studies of MHV (74,75) and Theiler's murine encephalomyelitis virus [TMEV] (76,77). The basic method involves digesting labeled virion RNA with T1 RNase which specifically cleaves at guanosine residues. Polynucleotides of twelve or greater base residues are then resolved based on their composition and chain length by two dimensional electrophoresis in polyacrylamide. Care must be exercised in the interpretation of results, however, since the method detects only 10 to 15% of the viral genome. Thus, differences in fingerprints may be construed as being indicative of differences in genomes, but identity of fingerprints means that only that portion of the genome being measured is identical. Rapid evolution of RNA viruses, as with polio virus during epidemics, is the chief limitation of the technique, since distant relationships may go undetected. A recent advance, reported for TMEV, is the analysis of RNA extracted directly from animal tissues (77). This eliminates the need to grow the virus in cultured cells, a step which could increase the risk of selecting a variant virus population.

Restriction enzymes have been useful for mapping genomes of DNA-containing viruses and for defining differences between closely related strains of a single virus. The method has gained routine clinical application based on its ability to distinguish between HSV types 1 and 2. Endonuclease digestion was one of the earliest molecular methods used to compare the prototype and immunosuppressive strains of MVM (78).

Genetic conservation can also be studied by nucleic acid hybridization. Using electrophoretically separated segments of double-stranded RNA viruses, it is possible to determine for each segment (gene) the extent of genetic homology. This method has been applied to several genera within the reovirus family (reviewed in 79) and may, in the future, contribute to our understanding of the epizootiology of rodent rotaviruses.

Monoclonal antibodies can be powerful tools for the discrimination of viruses and/or strains that are indistinguishable by other methods. For example, until the 1960's it was virological dogma that there was no antigenic variation among laboratory strains or field isolates of rabies virus. With the identification of several African viruses distinct from but related to rabies virus (80), this dogma was questioned. Just a bit more than a decade later, application of hybridoma technology to the question suggests that there are minor differences in the antigenic structure of rabies virus strains (81). Monoclonal antibodies also have the potential to distinguish between wild type and vaccine virus-induced infections. This approach has recently been reported for the distinction of poliomyelitis resulting from wild type virus vs. the Sabin vaccine (82). The application of monoclonal antibodies to diagnostic problems may be limited by their extraordinary specificity, but the use of pooled hybridoma products may overcome this limitation.

As we learn more about the biology and epizootiology of rodent virus infections, we may find that some of the methods discussed here must be applied to the standardization, monitoring and safety testing of vaccines that will inevitably be developed. Discussions of vaccines as control agents for rodent viruses have already begun. Human virology teaches us, however, that we must approach the issue of control with caution. We must first adopt standards for use in our laboratories. While a preliminary effort has been made in that direction (83), more needs to be done. An exercise recently published on polio neutralizing antibody testing serves as an excellent example (84). Sera were distributed for testing to 20 laboratories in twelve countries. Analysis of the results revealed greater than ten-fold variation in sensitivity of the tests used. Based on these results, a protocol was proposed for large scale determinations of polio virus antibody. It was also suggested that antibody titers be expressed in international units relative to a reference standard to obviate the inevitable problem of laboratory variation. Similar standard guidelines have been adopted by laboratories performing rabies virus diagnosis.

Prior to the institution of control measures and/or vaccination for infectious agents in the human or animal situation, several biological characteristics must be considered. Are there multiple strains of the agent that afford no significant cross-protection? How long is the infected host capable of transmitting infection to suscep-

tible contacts? How many potential routes of transmission exist? And a question which is particularly relevant to the laboratory animal situation: would vaccination result in the same types of interference (e.g., immunosuppression) induced by wild type infection?

The field of diagnostic virology is currently in a state of transition. This statement is particularly true for the laboratory animal facility, which frequently extrapolates from technological advancements in human diagnostic laboratories. However, we must keep in mind the differences in the populations of interest and the impact of disease on these populations. These considerations will, no doubt, modify both our objectives and our course of action. In view of the relative immobility and accessibility of the population of interest, we may find that our long-term goal should be to develop programs which emphasize eradication and facility modernization rather than those which support further development of diagnostic technology and vaccines.

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