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Recent Advances in Rabies Diagnosis and Research

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In Canada, rabies is a reportable disease under the Federal government's Animal Diseases and Protection Act. Administration of this act by Agriculture Canada has led to fairly uniform procedures for field investigations, laboratory diagnosis, reporting of laboratory findings and, to a certain extent, control of this disease in domestic animals. Although there are occasional exceptions, the main steps that occur in suspected cases of rabies are as follows: the practicing veterinarian or owner notifies a District Veterinarian of Agriculture Canada's Veterinary Inspection Directorate; the District Veterinarian investigates the case and, when appropriate, submits specimens to a laboratory of the Animal Pathology Division. Diagnostic tests are done and results are reported to the District Veterinarian who, in turn, notifies the owner, the Medical Officer of Health and others involved in the case. The district Veterinarian also imposes quarantines, authorizes payment of compensation, arranges for vaccination clinics when warranted, and generally keeps the public informed about rabies in the area.

Various provincial and municipal agencies are involved in, and affected by, the above activities. These agencies also administer provincial and municipal laws concerning rabies, especially quarantine of animals that have bitten humans and control of rabies in wildlife. Some provincial governments, namely those of Ontario and Alberta, have extensive research programs on the control of rabies in wildlife.

LABORATORY DIAGNOSIS OF RABIES

The primary test used for the diagnosis of rabies in most industrialized countries is the rabies fluorescent antibody (RFA) test (1) on brain tissue. This test can be completed in approximately 2 h and is highly accurate when done routinely by experienced personnel. It is used on all suitable rabies-suspect specimens submitted to any of the Canadian rabies diagnostic laboratories (currently the Animal Diseases Research Institute, LETHBRIDGE, Alberta and the Animal Diseases Research Institute, NEPEAN, Ontario). A secondary or back-up test, the mouse inoculation (MI) test (2), is used on human contact specimens that are negative on the RFA test. Observation of mice for 30 days is required to confirm a negative RFA test. Currently less than 0.1% of RFA-negative specimens are positive on mouse inoculation (3). This compares favourably with 7-15% false negatives when the primary test consisted of a Negri body strain on brain smears (established from the records of the Animal Diseases Research Institute, Nepean, 1955-1964). Although the RFA test is highly accurate, the long delay required for completion of the MI test causes considerable anxiety for exposed persons and health officials.

Replacement of the MI test by viral isolation in tissue culture may soon be feasible. Although early attempts to isolate street virus in tissue culture gave inconsistent results, recent studies using improved isolation techniques suggest that this method is at least equal to and perhaps has greater sensitivity than the MI test. Following further studies, we hope to recommend that the MI test be replaced by tissue culture isolation

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within one year. This will have the important advantages of shortening the observation period to three or four days and eliminating the need for routine inoculation of about 30,000 mice annually for rabies diagnosis in Canadian laboratories.

In addition to the above routine tests, human skin biopsies, corneal smears or other samples are occasionally examined by the RFA technique. In cases where paraffin-embedded tissues are the only specimens available, we can still use the RFA test after sections have been treated with trypsin (4). However it is not as sensitive as the RFA test on fresh tissues and, therefore, should not be used for routine diagnosis.

RABIES MONOCLONAL ANTIBODIES

Since the initial production of rabies monoclonal antibodies by Wiktor and Koprowski (5), additional panels (of antibodies) have been produced in the United States (6), Germany (7), France (8) and Canada (personal communication, R.B. Stewart, Queen's University, Kingston, Ontario). These antibodies may be produced in tissue culture or mouse ascitic fluids and can be used in many common laboratory tests devised for polyclonal antibodies.

Prior to the development of monoclonal antibodies most rabies street virus isolates were considered to be antigenically homogeneous. However, testing with monoclonal antibodies (Supplied by Dr. T. Wiktor, Wistar Institute, Philadelphia and J. Smith, CDC, Atlanta, Georgia) revealed many different antigenic profiles of street viruses and vaccine strains. "Typing" street virus isolates is proving to be valuable in epizootiological studies since in some cases the progression of particular strain(s) of virus can be traced through animal populations and geographic regions. Vaccine-induced rabies can be diagnosed by testing virus from a suspected case with monoclonal antibodies. In a field trial of an oral vaccine for wildlife in Ontario, we are monitoring virus isolates from animals in the target area.

Canadian street virus isolates have been examined with a panel of antinucleocapsid antibodies of ADRI, NEPEAN (9). Further studies have revealed four antigenic groups in terrestrial mammals: one major group in eastern Canada (infecting all species of terrestrial mammals and which is a southward extension of the rabies found in the Canadian Arctic); a second smaller group in a limited geographical area in southern Ontario; a major group in the southern portions of Manitoba, Saskatchewan and Alberta (represents the northern extension of "skunk rabies" from the mid-central U.S.A.); and a fourth very small antigenic group found in skunks in the Brooks, Alberta area. Four different major antigenic groups are found in bats in Canada and these are based both on host species and geographical areas.

Other uses of monoclonal antibodies include selection of rabies virus mutants, and studies of the pathogenesis including virulence factors and immunogenicity related to specific antigenic determinants. Some of the mutants selected in this manner are avirulent and are being considered as candidate vaccines for vaccination of wildlife (10). Mutants may be selected not only *in vitro* but *in vivo* suggesting a mechanism of antigenic variation in nature (11). The role of monoclonal antibodies in studies of the pathogenesis will be discussed later.

RESEARCH ON RABIES CONTROL IN WILDLIFE

In Canada, the United States and Europe, the main source of rabies in domestic animals continues to be certain wildlife species that support enzootic rabies. Although vaccination and other control methods for domestic animals reduces economic losses and the number of human exposures, substantial control or eradication eventually must depend on what we do about rabies in wildlife. During the past 20 years, research on control of wildlife rabies has centered on development of oral rabies vaccines (12,13,14). Countries that have been especially active in this field are Canada, the United States, Switzerland, West Germany and France. Switzerland, West Germany and Canada currently have field trials of a live vaccine in progress. The vaccine appears to be successful in areas where the fox is the target species (14).

In Canada, research on oral vaccines for wildlife has been funded almost entirely by the government of Ontario. During the past 17 years, a research group of the Ontario Ministry of Natural Resources has worked on development of baits and bait distribution, and the ecology of wildlife vectors. From 1968 to 1973 the Ontario Ministry of Health funded research at Connaught Laboratories on oral rabies vaccines. Recently the Ontario Ministry of Natural Resources has funded an intensified research program on oral rabies vaccines. The Rabies Advisory Committee (RAC) was established in 1979 by an Order-in-Council of the government of Ontario. Since then, the committee has been directing research to develop a suitable rabies vaccine, and an effective delivery system for vaccinating wild animal populations. Grants were awarded to scientists at several institutions to conduct research on various facets of oral vaccine development. The institutions and the main activities were as follows: Queen's University (computer modelling, production of a pathogenic strains of rabies virus and monoclonal antibodies); University of Toronto (antibody testing, preparation and testing of vaccine-containing capsules, genetically- engineered vaccines, testing of adjuvants); University of Guelph (immune response in foxes); University of Saskatchewan (testing vaccines and immune response in skunks); Connaught Laboratories (development and production of vaccines and baits, experimentation with adjuvants, potency and safety testing); Ontario Ministry of Natural Resources (computer modelling, wildlife ecology and development and testing of baits); Agriculture Canada (challenge trials, testing experimental vaccines in skunks and foxes).

Both live and inactivated vaccines were tested in foxes and skunks. Although researchers made some progress with inactivated vaccines given directly into the intestine, generally the proportion of vaccinated animals that became immune was too small to warrant use in the field (15). This method of vaccination poses the additional challenge of developing an effective method of encapsulation to allow the vaccine to pass through the

stomach with no loss in immunogenicity. In foxes, some live vaccines including commercial ERA® were effective when given for absorption through mucous membranes of the mouth or intestine (15,16,17). To date, similar standard live rabies vaccines have not been consistently successful in skunks. On the other hand, results of preliminary studies at the Animal Diseases Research Institute, NEPEAN, of a vaccinia virus recombinant containing the rabies virus glycoprotein gene (18) are encouraging. Moderate to high titers of serum neutralizing antibodies were producedin skunks given the recombinant in baits, by endoscope, by intramuscular inoculation and by scarification. Work continues to develop a safe vaccine that is efficacious in both foxes and skunks.

In other areas of the program, major achievements have been made in developing a bait that is acceptable to a high proportion of foxes and skunks, a bait distribution system (19) and a computer model of rabies in wildlife (20,21,22). The computer model will be useful in predicting outbreaks of rabies, in estimating levels of immunity to control rabies, and in evaluating the results of field trials.

In summary, the progress being made in development of vaccines that are immunogenic by the oral route and the marked improvements in auxiliary systems are encouraging signs that vaccination of wildlife in their natural habitats will eventually be successful. A field trial is in progress in Huron County Ontario. This trial is intended to test several features concerning the bait, the bait distribution system, acceptance of bait by wild animals, and the efficacy of ERA® in wild free-ranging foxes.

PATHOGENESIS

Our knowledge of the pathogenesis of rabies comes mainly from work done since the late 19th century. The following is a brief description of some of the important features of this process. Early studies established the neurotropism of the agent and the infectious nature of saliva of rabid animals (23,24). Subsequent studies established the following general steps in the movement of virus through the animal body: 1) introduction of virus into a bite wound or laceration; 2) migration via peripheral nerves to the central nervous system (CNS); 3) spread through the CNS; 4) centrifugal neural transport of virus; and 5) infection of nonnervous tissues (25,26,27).

One of the concerns with the inoculation site has been to determine whether virus enters peripheral nerves directly without preliminary replication in nonnervous tissue, or indirectly after replication in nonnervous tissue. In some experiments, nerve resection or limb amputation proximal to the inoculation site was lifesaving for only a short period after inoculation (28,29), indicating that virus could enter peripheral nerves directly without preliminary replication in nonnervous tissue. Similarly, studies using a street virus with a long incubation period suggested that, in some cases, virus could be retained for prolonged periods at the inoculation site (30). These latter findings were compatible with a period of replication in nonnervous tissues. Immunofluorescence and electron microscopic studies demonstrated that muscle fibers at the inoculation site in hamsters and skunks could be infected directly by rabies virus in the inoculum (31,32) and, thus muscle could be a site for preneural replication of virus. The relative importance of these two mechanisms (direct and indirect entry into peripheral nerve) has not been established. The neural route to the CNS was demonstrated by experiments using nerve resection and from evidence that antigen occurred in the CNS first at sites connected by nerves to areas of inoculation of virus (25,28,29). Other evidence indicated that this centripetal movement in peripheral nerves was in axons via retrograde axoplasmic flow (33,34). Electron microscopical studies demonstrated that viral replication in the CNS occurred almost entirely in neurons (35) and that cell to cell transfer of virus (transneuronal dendroaxonal transfer of virus) occurred by a process of budding on perikaryal and dendritic plasma membranes with simultaneous viropexis by adjacent axon terminals (32,36).

Following dispersal from the CNS, virus replicates in some nonnervous tissues. In the salivary glands, replication of virus in epithelial cells was accompanied by abundant budding on apical plasma membranes — thus accounting for release of virions into glandular ducts and saliva (37). In naturally infected skunks, several salivary glands and nasal glands support growth of rabies virus (38). The mechanism of infection of salivary gland epithelial cells involves primarily neural-epithelial cell transfer of virus rather than cell to cell transfer among epithelial cells (39). There is evidence that in skunks, viral titers in the submandibular salivary gland may be markedly influenced by the immune response (40,41). Unpublished studies in our laboratory suggest that the immune response impeded neural-epithelial cell transfer of virus.

Recent studies have focussed on the roles of cellular receptors and viral antigenic determinants in the pathogenesis of the disease. It has been suggested that acetylcholine receptors are important in the initial neural uptake of rabies virus at the inoculation site (42,43,44,45,46,47). Others have demonstrated that acetylcholine receptors are not essential for infection of rat myocytes and other cells in culture (48). Tsiang (49) suggests that the cellular receptor for rabies virus is not a unique specific molecule but is more likely a complex structure involving many cellular components.

Monoclonal antibodies are proving useful in studies of the pathogenesis and, possibly, in the development of vaccines. Antiglycoprotein monoclonal antibodies were used to select mutants of challenge virus standard (CVS) virus that no longer reacted with the antibodies used for selection. Some of these mutants were apathogenic when given to mice by the intracerebral route (50,51,52). The loss of antigenic sites on the glycoprotein molecule and associated virulence has been traced further to substitutions of specific amino acids (53,54). As stated previously, tests are underway to determine the efficacy of such mutants when used as vaccines (10). The above findings complement those of Wunner and coworkers (55) who have identified regions on the glycoprotein molecule that are responsible

for different types of immune responses. These areas of investigation should eventually lead to much greater precision in the design of vaccines, increased understanding of the immune response to rabies and, possibly, to improved treatment of clinical cases.

Recently we reported spongiform lesions in brains of infected animals (56). These lesions were detected first in experimental rabies in skunks and foxes, and later in naturally occurring rabies in the following species: fox, skunk, cow, horse and sheep. Vacuoles, 1-60 μm occur in the neuropil of the grey matter, only rarely in neuronal perikarya. This "spongiform change", as defined by Masters and Richardson (57), is a cardinal feature of the traditional subacute spongiform encephalopathies: scrapie, transmissible mink encephalopathy, kuru, Creutzfeldt- Jakob disease, and wasting disease of mule deer. The lesions in rabies are considered to be etiologically distinct from the subacute spongiform encephalopathies, since the incubation period in rabies is much shorter and the lesions occur only in rabies-positive animals. They were not detected in control skunks or foxes or in animals inoculated with rabies virus that failed to develop clinical signs. Also, in preliminary studies, we have not detected spongiform change in mice or rats experimentally infected with CVS rabies virus or street virus. The experimental disease in foxes and skunks may be useful to study the comparative pathogenesis of spongiform change since the rabies virus is fairly well characterized and the lesions occur in a high proportion of animals that have rabies encephalitis.

CONCLUSION

Many of the recent advances in rabies diagnosis and research have been made in the fields of advanced biotechnology, especially genetic engineering, biochemistry, immunocytochemistry, and monoclonal antibody production and testing. In the future it may be possible, through a combination of mutant selection and genetic engineering, to design vaccines that are totally apathogenic and that meet requirements for specific types of immune responses and selected routes of administration. Studies of the pathogenesis are likely to lead to determination of the cellular receptor(s) for rabies virus, the nature of the long incubation periods, the mechanisms of salivary gland infection, and factors that are necessary for recovery from CNS infection. All this should contribute to the development of better vaccines, postexposure treatment and even treatment of clinical cases of rabies. Such achievements will be attainable mainly through a blending of several fields of advanced biotechnology with classical methods of investigation. Many facets of rabies work such as field investigations, border control, laboratory diagnosis and basic and applied research still require use of proven procedures and well established technology. This requirement for the "old" methods is likely to continue, so that in many instances adoption of the products of genetic engineering and other technological advances will require expansion rather than a change in work from one technological field to another.

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