RABIES

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PREFACE

Rabies is an ancient disease and a fearsome one. Although it may not have the economic or public health importance of some other infectious diseases, few are so well known or carry the same emotional impact. Mainly transmitted by the bite of an enraged animal, and with practically no hope for recovery among those afflicted, it has provided the substance of stories and legends throughout the ages.

The pioneering work of many 19th century workers, culminating in the development of the first rabies vaccines by Louis Pasteur, provided the ground work for the modern era in the study of rabies. Since then, and particularly in the last quarter century, considerable advances have been made in our knowledge of the nature of the infectious agent, its mode of transmission and pathogenetic mechanisms. Yet even today, much remains to be learned about the disease. For example, although effective vaccines exist for humans and other animals, there is still no known practical cure once the neurological disease symptoms develop. Markers of virulence have been mapped at the molecular level, but it is yet unclear as to how rabies virus actually exerts its pathological effects.

The aim of this book is to provide a highly contemporary account of what is known about the nature of rabies (and related) viruses, what these viruses do, and what can and might be done to control or eradicate them. In recent years, compelling opportunities to answer these questions have been provided by advances in immunology, molecular biology, computer modelling, immunocytochemistry, and the neurosciences. Ongoing work in these areas should lead to much greater precision, economy, and safety in designing new vaccines, a far better understanding of the pathogenesis and epizootiology and, possibly, even to effective treatment of rabies encephalitis. Field trials of oral rabies vaccines have already demonstrated that it is possible to eradicate the disease from fairly large geographic areas. Monoclonal antibody testing and ecological studies will continue to contribute to epizootiological data and this, in turn, to the computer modelling needed to assess the risk of spread of the various types of species-specific enzootics and to monitor control programs. The ability to modify the virus in predictable ways, as a result of increasing

knowledge of the molecular aspects of the virus and nervous system, should provide additional refreshing insights into the mechanisms of cellular tropisms and other aspects of virus-cell interactions. Rabies is an example of a disease in which the immune system is compromised by one of the worst possible scenarios - in most instances, lack of induction until infection has become well established in a vital, non-regenerating tissue. Studies of the immune response (with particular emphasis on execution in neural tissue) might provide essential clues about the body's methods of combatting infectious neurological diseases, and eventually, could lead to an effective treatment of clinical rabies.

In this volume, one in the series "Developments in Veterinary Virology" of Professor Yechiel Becker, emphasis has been placed on the study of the disease in animals other than man. Nevertheless, by virtue of its broad scope, it is intended that the volume should be of interest not only to veterinary virologists but also to basic researchers, wildlife biologists, veterinary inspectors, health care workers, and others wishing an overview of the disease.

RABIES

1

UNDERSTANDING THE NATURE OF RABIES: AN HISTORICAL PERSPECTIVE

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Throughout recorded history there have been major epidemics and epizootics which have decimated populations and influenced the course of events. Man has been particularly affected by bubonic plague and smallpox and, less easily traced until modern times, influenza. Among man's domestic animals the plagues of cattle, whether rinderpest or bovine pleuropneumonia, at their worst caused starvation and hence lowered resistance in human populations; they frequently preceded severe epidemics, most notably the Black Death.

By such standards rabies pales to near insignificance. Dogs, wolves and foxes have been the most important hosts in European outbreaks; they have survived as species without difficulty, and the number of human victims has never merited the attentions of demographers. Yet the disease can be traced with certainty, in popular and in medical literature, further back in time than any other infectious or contagious disease. Its alarming manifestations in man and dog alike, the distressing course of the clinical disease and its almost inevitable progression to a fatal outcome have ensured unparalleled notoriety and unparalleled attention from both lay and scientific authors.

The "mad dog" was mentioned with respect and concern in the legal documents of Mesopotamia in the 23rd century B.C. where one may notice that the owner of a biting dog causing the death of a slave was liable for little more than a third of the compensation due in cases where the victim was a free man. Already in the Egypt of the Pharaohs the disease may have been causally linked to the saliva of the dog in magic incantations; and in ancient Chinese writings there are indications that rabies in dogs was recognized centuries before the birth of Christ (1).

J.B. Campbell, K.M. Charlton (eds.), RABIES.

It was also realised at an early date that a very wide range of animals was susceptible to the disease. In the *Historia Animalium* Aristotle, in the 4th century B.C., stated clearly that the bite of a dog mad with furious rabies would transmit the disease to animals of all other species, with fatal results although for unexplained reasons he had reservations about the susceptibility of man (2). Celsus on the other hand, writing in the first century A.D. had no such reservations. He wrote on preventive measures: "Especially if the dog was rabid, the virus must be drawn out with a cupping glass". He also recommended cauterising the wound and keeping it open "in order that the virus may run freely from it". It should be remembered that in the Latin of Celsus's day the term "virus" denoted "poison", with the added connotation of "slimy" - an apt enough description of rabid saliva in an age which could have no conception of the meaning the word would have for Pasteur in the 19th century, let alone its present day identity (3).

Celsus's near-contemporary Pliny had little of originality to say on the subject, but his writings reflect the views of therapy which were to prevail for many centuries, as well as the supposed prophylactic measure of removal of the "worm" from the tongue of puppies to prevent them from ever developing madness. This in its turn would seem to presuppose the long-held belief in a spontaneous origin of the disease induced by extremes of heat, drought, sexual frustration, and other stressful conditions which survived even alongside the realisation of transmission by saliva until at least the 19th century. Instead of Celsus's rational cautery Pliny offered various therapeutic recommendations including applying to the wound ash from the burnt head of a dog, or axle-grease pounded with lime (both owing something to cautery). Wholesome drinks included decoctions of dung of badger, cuckoo and swallow and the cast slough of snakes pounded in wine with a male crab, and, rather more palatable and "recently revealed by an oracle", the root of the dog rose (4).

Galen at the end of the 2nd century A.D. believed that only the dog species was naturally "receptive" to rabies, and that the disease so corrupted their humours that a mere drop of saliva falling on the hand of a man could infect his whole body with its poison (5). Greek and Roman teaching came together when Caelius Aurelianus, probably in

the 5th century A.D., produced a Latin version of the writings of Soranus of Ephesus (*fl.* 1st - 2nd century A.D.) on acute and chronic diseases. Soranus in turn leaned on texts by Democritus (C 460 - 370 B.C.) which were later lost in the destruction of the great library at Alexandria. The treatise included a chapter on hydrophobia, and 19th century writers made capital of a sentence which suggested to them that the author considered involvement of the nervous system in the pathogenesis of rabies. Drabkin, responsible for an English edition of the text published in 1950 pointed out that there is no reason to believe that "nervus" in Latin or "neuron" in Greek at the time of the pre-Socratic philosophers meant anything more than "sinew". In any case Caelius (or Soranus) did not think that Democritus distinguished clearly between hydrophobia and tetanus (and the diagnostic difficulty remains to this day), and credited Gaius, a follower of Herophilus, with priority for identifying "the brain and its membrane" as the parts affected in rabies (6).

The classic descriptions of the disease, and the classic recommendations for prevention and therapy, were repeated in many later works. Vegetius, more concerned with horses, mules and cattle included an antidote for cattle wounded by a mad dog in his *Ars Veterinaria* in the 5th century; Paul of Aegina in the 7th century felt the need for detailed descriptions of the disease in both man and dog "because these animals are numerous and domestic, and are frequently seized with madness". Unlike Caelius, but in agreement with such earlier authorities as Galen and Dioscorides, and later Avicenna, Paul of Aegina favoured the use of white hellebore in "antidotes" (7). He also noted a "redness of the whole body, but especially of the countenance" as later described by Avicenna in the 11th century.

In Britain mad dogs and legal problems pertaining to the killing of dogs supposedly mad were mentioned in the ancient laws of Wales compiled by Howel the Good (Hywel Dda) in the 10th century. Thus someone killing a dog because of its madness must be able to "prove it by showing that he saw him fighting with dogs and men, or that he saw him with his tongue greatly inflamed" (8). This may not carry great weight as a differential diagnosis; but the inflamed tongue brings to

mind a splendid colour plate of the tongue of a rabid dog published in London by George Fleming in 1872 (9).

Although descriptions of what was almost certainly rabies in dogs and in man (the latter usually referred to as hydrophobia) abound in early literature, from classical antiquity through Europe's Dark Ages and the Arab's revival of the medical classics, understanding of the nature of the disease made little progress until the 16th century. In 1546 Girolamo Fracastoro (1478-1553) published at Venice the first known treatise devoted exclusively to contagious diseases. Nutton has cogently and persuasively argued that Fracastoro's (purely theoretical) concept of contagion and of "seeds of disease" was not as original as has often been claimed by his admirers, and has demonstrated a number of sources and influences unacknowledged by Fracastoro, from Lucretius's disease-causing "seeds" to Galen's brief consideration of "seeds of disease", which may owe something to the Pre-Socratic atomists (10). Whatever his sources Fracastoro certainly developed such received ideas to no small extent, nowhere more so than in his chapters on specific diseases.

His chapter on rabies marks an advance in understanding of the disease, a halfway point between, on the one hand, Caelius and Paul of Aegina, and at the other extreme, the developments of the 19th century. Here acknowledging his sources in general with the sweeping prefix "all agree" he emphasised that it is not possible to contract rabies by simple "contact, or by fomes, or at a distance, but only when the outer skin is so torn by the bite of a dog that blood is drawn; as though contagion takes place in the blood itself through contact with the teeth and foam from the mouth of the rabid animal". As for pathogenesis, Fracastoro believed that rabies, like other contagions (and his choice of examples included scabies, syphilis, phthisis, "pestiferous fevers" - and "the rest") were caused by production in the animal body of putrefaction "both foul and confined" from which arose germs which were transmitted to other individuals to begin again a cycle of putrefaction and germ production. But his vision of germs "with the power to propagate and engender what is similar to themselves" did not embrace ideas of these "germs" or "seeds of disease" as *animalculae* or other living organisms. On the other hand he did state that animals

dead of the disease no longer "preserved the contagion" because "the germs of the contagion have perished together with the innate heat" - perhaps a dangerous assumption for the pathologist, were post mortems to be performed (11).

In spite of the felicity of his prose and the logic of his speculative thought, Fracastoro's treatise and the ideas it contained had no great influence in an age which lacked the means to demonstrate the validity or otherwise of its theses; an age in which, in the words of Nutton, "the hypothesis of causative seeds was a philosophical luxury for the intellectual practitioner" (12). The aetiology of rabies and of other contagious diseases continued to puzzle the medical profession and to baffle those searching for therapy for more than another three centuries; even then, in the case of rabies, post-exposure prophylaxis was to predate understanding of its aetiology by several decades. It would probably be unwise to become too excited at the passages where in Wright's otherwise sober and objective translation Fracastoro discusses the possibility of "immunisation" against pestilences - both the context and the Latin verbs used would seem to suggest that what he had in mind was more a process of developing a tolerance for the "germs" as is possible with certain poisons, e.g. arsenic (13).

During the centuries after Fracastoro the medical profession continued to be helpless in the face of the clinical disease; nor did the rise of a veterinary profession in Europe from the second half of the 18th century have any impact on the problem of canine rabies. This did in no way stem the flow of writing on the subject. Almost any kind of herbal remedy and other *materia medica* was recommended, and subsequently rejected, over the years. The authors concerned with rabies and with hydrophobia in man included many of the great intellects of the 17th and 18th centuries; even they could have little impact on either understanding of the disease, or therapy.

The pages of the *Philosophical Transactions* of the Royal Society contained many reports on the disease from its earliest years in the 1660s. Robert Boyle (1627-1691) wrote in 1666 on effects of sea water, especially in hydrophobia patients; Martin Lister (1638?-1712) wrote on rabies both in the *Philosophical Transactions* and elsewhere, noting

that the disease was less than common in England at the time, and that the dog was the principal carrier (14). In France Jacques Labessie de Solleysel (1617-1680), sometime Master of the Horse to the French Ambassador at the Peace of Westphalia, published a treatise on horses, their care and their diseases which went through a number of editions between 1664 and 1679. It is remembered today primarily for its clear and perceptive account of glanders and its transmissibility; but it contains also some advice on rabies in man, dogs and other domestic animals in addition to the horse. This in fact turns out to be an early example of two remedies which continued to be recommended well into the 19th century. Solleysel made no pretence of originality, or of first hand knowledge; one remedy was an unremarkable herbal concoction introduced in "a small book printed at Poitiers" by an author to whom it was given, as a favour to the general public, by a Jesuit in whose family it had been kept a closely guarded secret for centuries. The other was the powder of incinerated oyster shells to be made into an omelette for human patients, or given simply in olive oil to dogs, horses or cattle, one oyster shell sufficient for man and dog, but four or five required for horses or cattle. This, explained Solleysel, was a perfect substitute for those who were unable to get to the sea to benefit from the salt water treatment (15).

Solleysel may be described as a self-taught veterinarian in an age which had no possibility of formal veterinary education. He was a pioneer where glanders was concerned; his recommendations for rabies were strictly hearsay in an area of which he had no personal experience. They reflected attitudes which had been abroad since classical times. But elsewhere there were developments during the second half of the 17th century which were to provide the background to new attitudes which came into existence, slowly and gradually, during the next two centuries. The microscopes and observations of Antony van Leeuwenhoek (1632-1723) laid the foundations for the methodology on which bacteriology was eventually to be based. The ideas of Athanasius Kircher expressed in *Scrutinium Pestis* in 1658 may have been based on a misinterpretation of his microscopic observations, and his argumentation is not very clear; but he did formulate a case for an independent life of agents of disease. Finally, in the late 1680s, Francesco Redi

(1626-1697) disproved spontaneous generation of maggots in decaying meat, and his friends and associates Bonomo (d. 1696) and Cestoni (1637-1718) provided the first complete evidence for the causal rôle of the mite *acarus* in scabies in man. These observations led to speculation in the early 18th century concerning a *contagium vivum* as a possible cause of the cattle plague which was then decimating cattle populations in Italy and subsequently in the rest of Europe (16).

These developments had little impact on the bulk of rabies literature which rarely strayed into comparisons with the major epizootic diseases; but there was an isolated example of their influence although it was published anonymously and in a general, as distinct from a scientific, magazine. In 1735, in the 4th volume of the *London Magazine*, an anonymous author agreed with traditional opinions regarding the saliva of the rabid dog as the source of the disease; but he went further in describing this infectious medium as "minute particles or animalcula, mixt with saliva" which would insinuate themselves into the "nervous juice" and thus affect the brain. As there had never been any reliable reports of a cure where symptoms had appeared, the main objective must be to *destroy the animalcula* (my italics) before they could do any harm. His suggestions to this end were not innovative but included immersion in sea water and a diet strong in any kind of liver. With less than absolute confidence in remedies his conclusion was not designed to please dog lovers: prevention must "in great measure depend much on lessening the number of those animals, which produce such a terrible disease" (17).

Such bold linking of infection, let alone rabies infection, with Leeuwenhoek's *animalcules* was an isolated case even in the Century of Enlightenment. Three decades later, in Lyon and in Paris, were founded Europe's first veterinary schools, in 1762 and 1766, respectively. Their founder was Claude Bourgelat (1712-1779), a minor nobleman and enthusiastic rider and horse lover who in spite of efforts to educate himself found his avowed task of educating veterinarians competent to deal with diseases of all domestic animals - Europe was still in the grip of major cattle epizootics - difficult when those animals were not horses (18). His treatise on *materia medica* for the use of his students at Lyon published in 1765 is highly derivative and full of traditional

Figure 1. French broadsheet published in 1800, informing and warning the public of the dangers of rabies. By courtesy of the Wellcome Trustees.

remedies; and although he expresses contempt for those who believe in "the magic power of charms and mystic utterances" against rabies, he is happy to support the authenticity and efficacy of the remedy recommended by Solleysel in the previous century (19).

It is hardly surprising that diseases of horses and cattle took precedence over canine rabies in the works of the early veterinarians; the inroads made by glanders in horses and by rinderpest and contagious pleuropneumonia in cattle were of far greater economic importance in the 18th century than outbreaks of rabies. When these occurred, they seem to have been of greater concern to the medical profession worried about aspects of public health. D.P. Layard (1721-1802) who combined a country practice with a lively interest in the epidemiology of rinderpest and the possible benefits of inoculation, also wrote on rabies

following outbreaks in London around 1760, at a time when the authorities brought in regulations enforcing muzzling and restraining of dogs, and strict destruction of strays (20).

Towards the end of the 18th century there was a growing interest in research especially within the medical and scientific societies beginning to flourish in London and elsewhere. The year 1793 saw publications from two such societies which were of paramount interest to the future of rabies research. The societies in question were the Literary and Philosophical Society of Manchester, and the Society for the Improvement of Medical and Chirurgical Knowledge in London. In Manchester the author was Samuel Argent Bardsley (1764-1851). Born in Essex Bardsley studied surgery and medicine at Nottingham, London, Edinburgh and Leyden before becoming physician to the Manchester Infirmary in 1790. He was an active member of the Literary and Philosophical Society, and his observations on rabies and hydrophobia, later incorporated into a volume of case studies, are very remarkable for their time (21). For Bardsley was emphatic that there was no spontaneous occurrence of either rabies in dogs or hydrophobia in man, and that apparent cases of the latter unconnected with the bite of a rabid dog were due to other diseases and "sometimes hysteria". He argued cogently and well for the purely contagious nature of the disease, and having presented his evidence (including a reference to Hunter's paper discussed below) drew his conclusion which was not to be successfully put into practice until Pasteur had repeated the arguments nearly a hundred years later: "... it is upon the ground of having established an accumulated series of probable evidence that I erect the whole of the scheme for extirpating Canine madness from this island. The plan is as simple as I trust it will prove efficaceous. - It consists merely in *establishing an universal quarantine for dogs within the kingdom, and a total prohibition of the importation of these animals during the existence of such quarantine*." And he finally added: "Our insular situation is peculiarly favourable for the experiment" (*cf.* Pasteur, below).

The other paper of importance which had been published earlier in the same year appeared in the *Transactions* of one of the more exclusive of the London societies which had been founded in 1783 by the great

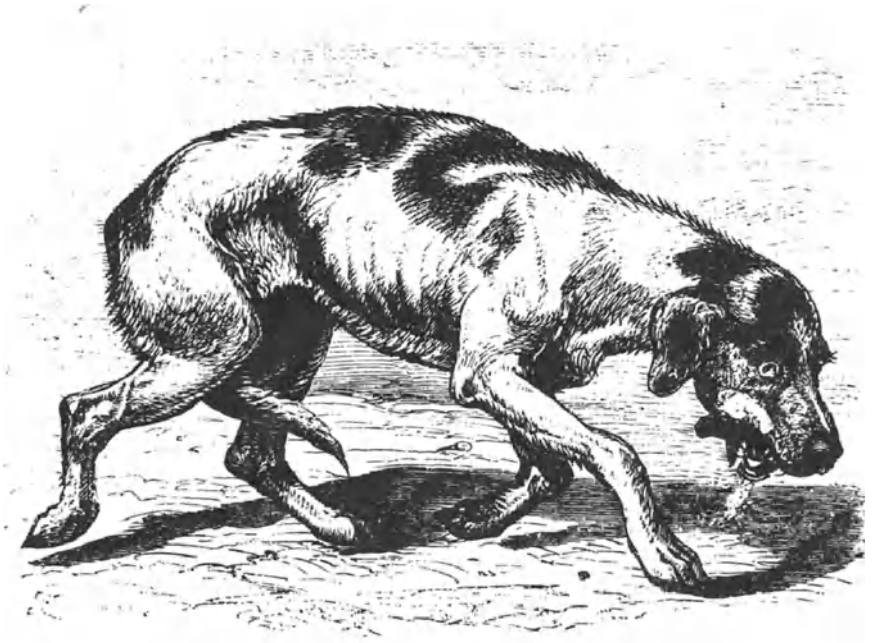

Figure 2. Furious rabies in the late stages. From G. Fleming, *Rabies and Hydrophobia*, 1872. Courtesy Wellcome Institute Library, London.

John Hunter, surgeon and anatomist (1728-1793), and a fellow Scot, George Fordyce (1736-1802). Its membership was limited to a total of 12 of which nine were founder members; all of them were close associates of Hunter's, and most of them had been his pupils. Only one volume of papers given at the society's meetings was published in Hunter's lifetime, and that in the year of his death (22). The penultimate paper of the volume dealt with "canine madness" and seems to have been the result of the combined efforts of the society's membership, although Hunter's younger namesake, John Hunter, M.D. (1754-1809) appears as the author (23). It is a paper full of sober reasoning which must be seen in the context of its time. The author admitted that he had no proof that rabies could not occur spontaneously, but that all his available evidence suggested that cases of the disease were commonly the result of infection. Personal experience in Jamaica had taught him that on an island full of dogs, in a hot climate, 40 years could elapse without a single case of canine rabies, and that such outbreaks as there had been in the past all appeared to

have resulted from introduction from North America. It is the concluding pages of the paper which raise it above the level of most writing on infectious diseases at this time, and which point the way to developments in the next century. They contain suggestions for experiments to be made "upon the poison" of rabies in order to explore its nature and path of transmission. Among the recommendations were inoculation of dogs, and other animal species, with saliva from dogs known to be rabid, or even from "an hydrophobic patient"; the inoculated dogs could then be observed and progress of the disease could be recorded. He also suggested experiments to determine the effects of "counter poisons", and of the time limit, if any, for excision of the site of inoculation to prevent development of the disease.

Neither Hunter himself nor any of his friends within the society appear to have thought of putting the suggestions into practice. Perhaps the death of *the* John Hunter in the October of the year of publication removed their inspiration. Ten years later the challenge was taken up in Germany, by Georg Gottfried Zinke (d. 1813). Zinke carried out all the animal experiments suggested, save the one involv-

Figure 3. Dumb madness. From G. Fleming, *Rabies and Hydrophobia*, 1872. Courtesy Wellcome Institute Library, London.

ing a human patient, and presented his results in a small volume published in Jena in 1804 (24). It is in fact the first record of a complete series of transmission experiments designed to demonstrate the path of the agent of rabies. Zinke succeeded in transmitting the disease by inoculating rabid dog saliva on a small brush into incisions made on the paws of healthy dogs, and in cats, rabbits, and fowl. He had read Hunter's paper and referred to it more than once in his book but did not otherwise acknowledge any influence of its suggestions on his own work. The inoculation experiment with saliva from a human case was carried out by François Magendie in 1813 but not published until 1821 (25) in a paper which contains also a graphic description of a dramatic inspection of rabid mastiffs in an establishment for fighting dogs in Paris. These early experiments did nothing to establish the identity of the disease agent, but the involvement of Magendie, pioneer neurophysiologist, reflects a growing interest in its neurotropic character. On the other hand there also continued to be those who warned of the dangers of experiments such as Magendie's. In London Benjamin Moseley had written darkly: "The source of rabid poison, in all animals, is unquestionably in the mouth. I have no doubt but deadly inoculation might be performed in a way, which I do not think prudence would justify the mentioning - There is mischief enough already in the world." (26).

In the late 1820s K.H. Hertwig (1798-1881) in Berlin made other ambitious transmission experiments when he attempted to induce canine rabies by implantation of nervous tissue from rabid dogs into healthy ones. None of six dogs treated in this way contracted the disease; in one case he succeeded by inoculation with tissue from salivary glands and in some others with rabid saliva (27). The results were perhaps less remarkable than the imaginative design of the experiments and the personality of their architect. Hertwig was indeed the first of those involved in rabies experimentation at this time to have availed himself of the educational facilities which now existed for those interested in comparative medicine. He had supplemented his medical degree, obtained at Breslau in 1819, with veterinary studies in Vienna, Munich, and Berlin, and after completing a further degree of doctor of medicine at Berlin taught at its veterinary school throughout his working life.

Throughout the 19th century Europe and the British Isles were plagued by frequent outbreaks of rabies which are reflected in the medical and veterinary literature of the time (28). The British veterinarian William Youatt (1776-1847) gave a course of lectures on "canine madness" which were published in *The Veterinarian*, the journal he co-edited with William Percivall (29), in the 1830s. In a manual on dogs written towards the end of his life and published posthumously, Youatt included a chapter on rabies which shows him to have adopted the views of Bardsley whom he quotes, although not unnaturally Youatt writes far more extensively on the disease in animals. Like Bardsley, Youatt believed firmly that rabies was a communicable disease, and that spontaneous occurrence was impossible; hence he also unreservedly adopted Bardsley's recipe for prevention: "... it would appear that if a species of quarantine could be established, and every dog confined separately for eight months, the disease would be annihilated in our country, or could only reappear in consequence of the importation of some infected animal" (30). He was more pessimistic than Bardsley regarding the possibility of enforcing such regulations, and he was very critical of the "number of useless and dangerous dogs" kept in the country, and most of all of the practice of keeping fighting-dogs for "the most brutal purposes". As for the nature of the "rabid virus" he admitted that knowledge was sparse, and that "it would be a difficult process to analyse it"; but he included a prophetic sentence: "I very much regret that I never instituted a course of experiments on the production and treatment of rabies in (the rabbit). It would have been attended with little expense or danger, and some important discoveries might have been made".

Less than 30 years later, the rabbit came into its own as the experimental animal of choice in rabies research. The use of the rabbit, which develops the predominantly paralytic form of rabies, was to provide the necessary basis for Pasteur's work on a vaccine. It was introduced to the world of science and veterinary medicine in Paris in 1879 by Pierre-Victor Galtier (1846-1908), professor at the veterinary school at Lyon where he had been educated, and where he spent all of his working life. In an essay on the history of rabies published in 1975 (31) J.H. Steele wrote of Bouchardat that he was "among the first

to think about inoculations against rabies and had an early influence on Pasteur. He attempted many experiments at the Lyon Veterinary Faculty". There is no documentation to support such a statement which would seem to stem from a confusion of the personalities of Bouchardat and Galtier. Apollinaire Bouchardat (1806-1886) was a pharmaceutical chemist attached to the Paris medical faculty and Hôtel-Dieu where he stayed as professor of hygiene until the year before his death at 80. There is nothing to suggest that he ever spent any time whatever in the Lyon Veterinary School. His connection with rabies came when he was asked, during one of the many 19th century outbreaks of the disease with a correspondingly alarming number of cases of human hydrophobia in France, to evaluate prescriptions for remedies submitted to the authorities. His reports were published in 1852 and 1855 and read as a catalogue of all the remedies which had been tried in vain through the centuries; and Bouchardat had no reservations about their uselessness (32). The only reference to inoculation in his reports comes in a comment on the second one made by the veterinarian Eugène Renault (1805-1863) who in 1852 had himself reported on the results of experiments in which he had inflicted bites by rabid dogs on a number of sheep, dogs, and horses (33). Renault told of an old man so convinced of the infallibility of a certain remedy that he had to be physically restrained from letting himself be bitten by a caged rabid dog; the "specific" later proved of no value in animal experiments.

Galtier on the other hand did carry out extensive experiments on rabies at Lyon, and pointed out the advantages of using rabbits the year before Louis Pasteur (1822-1895) turned his attention to the subject. Galtier's work may even have influenced Pasteur's decision to turn to the subject; his early experiments with rabbits and sheep certainly paved the way for the development of a vaccine. Having shown the possibility of transmitting rabies with dog saliva to rabbits in series, Galtier explained his intention of searching for "an agent capable of neutralising the virus of rabies after it has been absorbed and thus to prevent the clinical disease developing" (34). Two years later he had been able to show that he could immunise sheep which would become able to withstand challenge with inoculated rabid saliva by prior inoculation directly into the jugular vein. But by the time

these results were published, Pasteur had entered the arena. He had the advantage of better resources, a very well established reputation, and a number of extremely able collaborators. French historians of medicine have been at pains in recent years to set the record straight; Galtier does appear to have suffered some injustice, although this should not be allowed to diminish admiration for Pasteur's achievement (35).

During the years between 1879 and 1881 when Galtier carried out his initial experiments on rabies, Pasteur had established the principle of prophylactic inoculation with attenuated material in his studies on chicken cholera and anthrax (36). By the time he successfully demonstrated vaccination of sheep against anthrax at Pouilly-le-Fort in the early summer of 1881, he had been working on rabies for six months. He was certainly familiar with Galtier's publications; it has been suggested that he visited Lyon to further familiarise himself with the work there before beginning his own in December 1880 (37). From the beginning, he worked in close collaboration with Émile Roux (1853-1933), the only man with a medical degree in the group around Pasteur which included also Charles Chamberland (1851-1908); the veterinarian Edmond Nocard (1850-1903); and Louis Thuillier (1856-1883) who became a victim of the search for the agent of cholera during the outbreak at Alexandria. Both Galtier and Pasteur and his staff began with one clear advantage over earlier workers in the field of rabies research. The principle of specific agents causing specific diseases was at last a well established fact following Pasteur's own final refutation of spontaneous generation, and Robert Koch's development of techniques of staining and of pure culture which had led to the unequivocal identification of the anthrax bacillus only a short time before (38). But unlike the many agents being identified in rapid succession from the late 1870s onwards (39), no one had seen the "virus" or "microbe" of rabies, and no one had been able to grow it *in vitro*.

The impact of the discovery of the anthrax bacillus and the development of a vaccine against it may be seen by considering the opinions expressed by the London veterinarian George Fleming in a volume published less than 10 years before, in 1872. Fleming was critical of those of his own profession, Youatt among them, who had

earlier spoken out against spontaneous occurrence of rabies in dogs and who believed that "no transmissible disease ever arises spontaneously". To make his own position clear he added: "Whatever weight this line of argument may have in human medicine, there can be no doubt whatever that in comparative medicine it cannot be entertained as absolutely unassailable". He therefore devoted much space, with illustrations, to such means of prevention as the filing down of canine teeth and incisors, and the construction of muzzles which were adequately restraining while still allowing the dog to open its mouth "freely and widely" (40). It should also be noted that Fleming found it necessary to include a chapter on "Analogies and dissimilarities between rabies and anthrax", thus underlining the differences between the disease in man and in animals. In man the possibility of confusion, and hence comparison, has been with tetanus and its spasms, from the time of Caelius until the present day (*cf.* note 6 above). Now Pasteur was up against an infectious agent he could neither see nor cultivate in the laboratory; undeterred he grew it, and attenuated it, in its natural habitat, the central nervous system of his laboratory rabbits. This was finally possible after long and hard experimentation had established two fundamental facts. One was the neurotropic character of the

Figure 4. Dog with muzzle recommended for maximum convenience, from G. Fleming, *Rabies and Hydrophobia*, 1872. Courtesy Wellcome Institute Library, London.

virus, long suspected but only now proved conclusively. The other was the determination of a "virus fixe", a standardised form of the virus which unlike the street virus had a well defined incubation period. It was found that inoculation of street virus directly under the *dura mater* of dogs shortened the incubation period to no more than two weeks. The effect of the same virus passed through rabbits in series was intensified, and the incubation period correspondingly shortened, until a limit of six to seven days was reached. It was with this standardised virus that Pasteur and his coworkers went on to develop a vaccine. It required years of intensive work and countless animal experiments with dogs and rabbits. Even then, when the vaccine had proved its worth in dogs, Pasteur had to overcome a final agony of ethical indecision before inoculating, as a last resort, the badly bitten Joseph Meister (41). In spite of the severity of his injuries the boy survived without developing clinical rabies.

There were to be more successes in Pasteur's lifetime, and also occasional setbacks; but the principle of post-exposure rabies prophylaxis had been established. Interest abroad was great. In 1887 the British Government sent a commission to France to report on Pasteur's results. Its secretary was Victor Horsley (1857-1916), then Professor Superintendent of the Brown Institution where his work was frequently made difficult by the anti-vivisection lobby, a perennial problem for British animal experimentation. The visit proved to be of mutual benefit. The commission's report offered authoritative confirmation of Pasteur's results when he most needed it; the lesson learned by Horsley in particular resulted in the eventual eradication of rabies in Britain. Pasteur had for some time wanted to test a policy of dog vaccination, muzzling and quarantine as a means of stamping out rabies in an island community, and had considered the island of Mauritius as a suitable choice. Now the British Isles offered an alternative closer to home. Horsley, with the political backing of Walter Long (1854-1924) achieved eradication by 1902, and could use this fact as part of his ammunition when he testified before the Royal Commission on Vivisection in 1908, although he had in the meantime lost a laboratory assistant, bitten by a rabid cat, who died in spite of treatment by Pasteur in Paris (42).

The successful development of a vaccine, and the resulting profusion of vaccine institutes established in rapid succession in France and elsewhere, left unanswered the fundamental question of the nature of the agent. In 1903 Paul Remlinger (1871-1964), after many difficulties because of the filter-clogging tendencies of suspensions of brain tissues, was able to demonstrate its filterability by judicious use of centrifugation and dilution of suspensions in combination with the largest pore size of Berkefeld filters. Like most of his peers he remained reluctant to accept M.W. Beijerinck's theory of a *contagium vivum fluidum* (43). The same year had seen the appearance of another source of confusion, similar to a problem already plaguing those studying the aetiology of smallpox. Adelchi Negri in Pavia described the inclusion bodies which still bear his name, identifying them as protozoa and claiming them to be the agents of the disease. He suggested a cycle of development, and the putative organism was named *Neurocytes hydrophobiae* (44). Negri bodies have remained an important diagnostic tool, but with regard to the search for an agent of the disease they were responsible for the laying of a number of false trails for decades after Negri's observations. As far as new knowledge of filterable viruses was concerned, the years between the two World Wars were characterized on the whole by work on ultracentrifugation and ultrafiltration, with the exception of attempts to crystallise tobacco mosaic virus. For rabies virus, ultrafiltration studies in 1936 indicated a particle diameter of 100-150 mμ, a tolerable approximation of the presently accepted average dimensions of 80 x 180 nm (45).

Following World War II work on tobacco mosaic virus, bacteriophages, influenza viruses, polio virus, etc., gradually built the framework of basic facts which, together with results obtained by the molecular geneticists, has informed our understanding of the biology of viruses (46). The necessary factual knowledge of the virion of rabies emerged in the early 1960s when a spate of studies established its chemical composition as an RNA virus and its morphology as the characteristic bullet shape, with a lipid containing outer envelope necessary for its infectivity (47). Already in 1918 Remlinger, innocent of the sophistication of knowledge to come within his lifetime, had found ether to destroy the infectivity of the virus (48). Analysis of the

Negri inclusion bodies showed them to contain RNA granules embedded in a matrix of DNA (49).

With this documentation of the morphology and chemical composition of the virus of rabies, understanding of its nature is if not quite complete at least becoming fairly well established. More recent results must be left to the reviewers of contemporary work, and the historian can turn for a conclusion from the stark medical and veterinary facts to certain legislative aspects which in less than 200 years have come full circle. Today western democracies are busily dismantling taxes on dogs claiming they have become administratively futile and uneconomic. A general tax on dogs was first mooted in 1810 in France in a volume on the history and prevention of rabies by a modest hospital administrator whose main claim to fame is as the father of Honoré de Balzac (1799-1850). Bernard-François Balzac (1746-1829) was no lover of dogs, as his text makes clear; the dog is described as a public enemy, of an "immoralité incurable". According to Balzac père the odours and emanations of these detested animals were the means of transmission of not only rabies but of other diseases as well, including plague. The benefits of dog to man were far outweighed by the ills it inflicted, and Balzac proposed a tax on dogs of from 3 to 50 francs, depending on the type of dog whether pet, hunting dog, watch dog, sheep dog, etc. The immediate impact of Balzac's treatise appears to have been minimal, and an official order on dog tax did not appear in France until 1855. One can only hope that there was a more positive response to Balzac's other main proposal which was in the form of an impassioned plea on behalf of patients who might only be suffering from psychological forms of hydrophobia. For their sake he also drafted a law intended to prevent a practice which had been given scant publicity, but which nevertheless had been widespread for centuries. It was the strangling or suffocating, usually by means of the sufferer's own bedclothes, of victims of this disease so terrifying to behold. It was of course done in the name of euthanasia, but Balzac feared it might prove "too tempting to the heirs or enemies of the patient". In this respect at least there is advance over the early 19th century. Such specific legislation is no longer called for (50).

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2

STRUCTURE OF RABIES VIRUS

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INTRODUCTION

Rabies virus possesses an unsegmented negative strand RNA genome. From a systematic point of view, all viruses presenting this particular genomic structure are grouped in 2 viral families, the Paramyxoviridae and the Rhabdoviridae (1). The host range of the Rhabdoviridae is surprisingly wide, extending from insects, with the sigma virus of Drosophila, to fishes and mammals (1). It is divided into 2 main genera, *Vesiculovirus* and *Lyssavirus*. The genus *Vesiculovirus* is comprised essentially of vesicular stomatitis virus (VSV) and its subtypes, and related viruses such as Chandipura and Piry. VSV is by far the best studied unsegmented negative strand RNA virus and has provided the basis for the majority of data accumulated concerning the viral architecture, mode of replication, and structural and functional studies (for a review see ref. 2). VSV is therefore a model for this group of viruses and will be referred to frequently in this chapter.

Rabies virus is the prototype of the genus *Lyssavirus*. This genus also includes the rabies-related viruses which have been isolated from African and, more recently, from European countries (3,4; see also King and Crick, this volume). These latter viruses are of particular interest since classical rabies vaccines may fail to protect animals against post-exposure to some of them (5,6). The first sequence studies to investigate the molecular basis of these antigenic variations are currently in progress in some laboratories, especially in the Wistar Institute, Philadelphia (Duvenhage) and the Pasteur Institute, Paris (Mokola).

Many of the rabies strains studied through the world have been derived from a rabid cow isolate by Louis Pasteur himself, a century ago (7). After several passages in rabbit brain, the virus became "fixed", and further adaptation to various host and cell types have led to the actual PM, CVS and PV strains (see ref. 8 for the precise story). "Fixed" viruses are characterized by their ability to kill animals, or to give optimal viral production in cell culture, after a very constant and predicable period. They differ from "street" virus isolated from naturally infected animals, in which the growth period and killing ability is very variable. Other fixed strains derived from independent isolates include the SAD and ERA strains from a dog, and the Flury LEP and HEP strains from a young girl (8; see also Bunn, this volume).

Despite the fact that rabies virus is frequently compared to VSV, some important features distinguish the 2 viruses. Primary among these are the viral tropisms, the rabies virus being clearly more neurotropic than VSV. Another important difference concerns the responses to viral infection *in vitro*, the VSV inducing a clear inhibition of cellular macromolecular synthesis (9) whereas the rabies virus shows either little (10) or no (11) inhibitory effect. Furthermore, the rabies virus cycle is substantially slower than the VSV one (12) and viral production is lower (11). Therefore, the recent determination of the complete sequence of the rabies genome in our laboratory (13-15) was a necessary step for 2 reasons: firstly, to test if the differences observed in the biology of both viruses were understandable at the molecular level by comparison with the genomic sequence of VSV (2); and secondly to produce a library of complementary DNA clones usable as probes to detect viral genes or gene transcripts, either in infected cells or in infected animal tissues. The latter is the more promising tool to date, and its action spectrum extends from fundamental studies of the viral biology and pathology, to the diagnosis of rabies.

MORPHOLOGICAL STUDIES

The first attempts to visualize the rabies virus by electron microscopy were undertaken in the 1950s and concerned examination of Negri bodies observed in the brain of infected animals (16-19). The

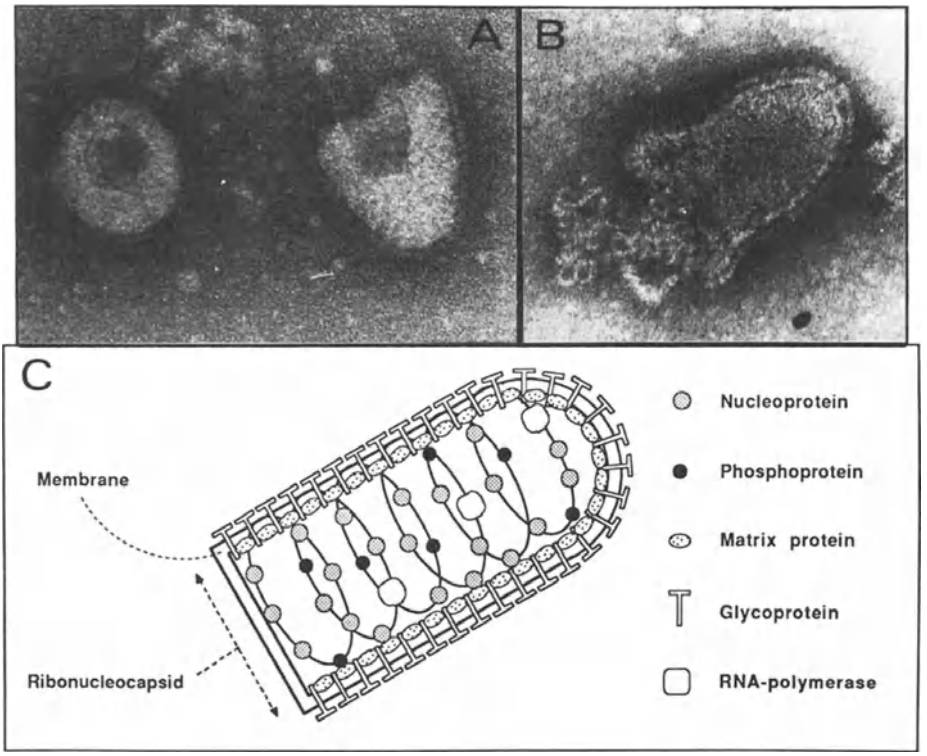

Figure 1. Structure of the rabies virus. **A:** complete virions; **B:** partially disrupted virions; photographed after negative staining electron microscopy (x 128,000). **C:** Schematic view of the virion.

technique of thin section electron microscopy, in 1962, permitted Roots (20) and Matsumoto (21) to report the first observation of the virus in the Ammon's horn of mouse brain. One year later, the adaptation of the virus to various cell cultures increased its multiplication rate, allowing its purification and leading to the first description of the morphological aspects (22,23). From that time, other studies have contributed to define precisely the viral morphology (reviewed in ref. 24).

By negative staining electron microscopy, the rabies virion looks like a bullet, with one end rounded and the other flat (Fig. 1A). Although the diameter of rabies particles is relatively constant, averaging 75 nm, the length is more variable and extends from 130 to

300 nm with a mean of 180 nm. Such a variation in the estimated length is due both to differences in the various virus strains studied and to the probable presence, in cell cultures, of defective interfering (DI) particles physically and antigenically indistinguishable from full length particles, but significantly shorter (25,26). These DI particles, which have been better studied in VSV (for reviews see refs. 27 and 28), usually possess a truncated genome. They are therefore defective in transcription and replication activity and require the presence of homologous infectious particles to assure their multiplication. Since their shortened genomes replicate rapidly, these interfere efficiently with normal genomes for encapsidation into the virion structures.

The virion is bounded by a lipoprotein membrane or envelope 7.5-10 nm wide, from which spike-like projections 9 nm long and separated by 5 nm intervals extend to the outside. The projections exhibit a knob-like distal extremity and are usually absent from the planar end of the particle. The viral envelope encloses an helical ribonucleocapsid forming a cylinder 50 nm wide and approximately 165 nm long. The periodicity of the helix being around 4.5 nm, 30 to 35 coils are necessary to form the total cylinder. In some degraded preparations, it is possible to observe partially disrupted virions in which the internal helix unwinds into a wavy ribbon from the planar end of the particle (Fig. 1B).

STRUCTURAL AND FUNCTIONAL STUDIES

The rabies genome codes for 5 proteins named N, M1, M2, G and L. The virus itself can be divided roughly into 2 structural and functional units, the viral envelope and the ribonucleocapsid core.

Chemical Dissection

Trypsin treatment of the native virion removes only the spike-like projections, leaving a small hydrophobic polypeptide firmly anchored in the viral envelope (29). This indicates that the viral spikes are formed from the external part of a transmembrane protein. In fact, 1 spike is formed by the association of the glycosylated (30-32) external extremities of 3 monomeric units of the glycoprotein G (33).

Treatment of the virion with nonionic detergents such as NP40, Triton X-100 or octylglucoside solubilizes the viral envelope (34,35) leading to the almost total extraction of the glycoprotein G. Two other viral proteins are also partially extracted, firstly the phosphoprotein M1 and then the matrix protein M2 (35). Nevertheless, experiments combining osmotic shocks and EDTA treatment have shown that only the matrix protein M2 is membrane-associated, anchored to the inner side of the viral envelope. The phosphoprotein M1, being in a more internal position, is most likely associated with the helical ribonucleocapsid (29,36,37).

Interestingly, the "core particle" released by treatment with nonionic detergents is functionally active in transcription (38,39). In the transcription complex, much evidence has been accumulated, largely from VSV studies (for a review see ref. 2), to suggest that the ribonucleocapsid-associated RNA-dependent RNA polymerase L encodes the majority of the viral enzymatic activities, such as RNA synthesis, capping, methylation, polyadenylation and at least part of the phosphoprotein phosphorylation. The phosphoprotein M1 seems mostly involved in regulatory functions and the nucleoprotein N is always strongly associated with the RNA genome, forming the required template for both transcription and replication. The solidity of the RNA genome-nucleoprotein N association is illustrated by the observation that only the helical ribonucleoprotein structure (RNP) is maintained after treatment of virions with an ionic detergent such as deoxycholate (34,40).

The above analysis allows the proposal of a schematic organization of the rabies viral particle (Fig. 1C). From this schema, it is apparent that all proteins exhibit mutual interactions, as has been shown by chemical cross-linking (29).

Quantitative Aspects

Each rabies virion contains a single molecule of genomic RNA. The number of copies of each viral protein in the virion was analysed some time ago, and it will be of peculiar interest to reassess these values with techniques such as dark field scanning transcription electron microscopy (41). On the basis of presently available data (30,42) there are respectively 1800, 950, 1500, 1800 and 60 molecules per

virion for the nucleoprotein N, phosphoprotein M1, matrix protein M2, glycoprotein G, and RNA-dependent RNA polymerase L. Molecular weights (MW) are unfortunately difficult to deduce from primary structures (13-15) since at least 3 viral proteins are known to be modified after translation, the N and M1 being phosphorylated (45-47) and the G glycosylated (30-32). From studies undertaken on different strains and under various conditions (43,44), however, the MW of the above proteins can be averaged to 57 (N), 38.5 (M1), 25 (M2), 69 (G) and 180 (L) Kdaltons.

The RNA Genome

The rabies genome is a single unsegmented RNA molecule of which the MW was first estimated by Sokol *et al.* to be 4.6×10^6 daltons (40). The complete sequence of the 11932 nucleotides has been recently determined in our laboratory (13-15). The genomic RNA is of negative polarity, indicating that it is unable to be infectious alone (40). Therefore, immediately after penetration into the cell, it must be transcribed into complementary positive sense molecules capable of producing viral proteins. This obligatory transcription step is assured by a genome-encoded enzyme, the RNA-dependent RNA polymerase, that virions must carry with them into the infected cell. The transcriptase activity associated with purified virions has been reported both *in vivo* (48) and *in vitro* (38,39).

Mechanisms of Transcription and Replication

Fig. 2 illustrates the mechanisms involved in transcription and replication. Transcription occurs from the 3' to 5' end of the genomic RNA template (49) and sequentially produces monocistronic transcripts: a small uncapped, non polyadenylated leader RNA (50) and 5 capped and polyadenylated messenger RNAs corresponding to the 5 known structural proteins of the virus (43,51-53). The leader RNA, varying in length from 55 to 58 nucleotides, is encoded at the exact 3' end of the genome (50). The order of the structural genes has been shown both by transcriptional mapping experiments (49) and by analysis of the nucleotide sequence (13-15) to be the nucleoprotein (N), phosphoprotein (M1), matrix protein (M2), glycoprotein (G) and the RNA-dependent RNA polymerase (L).

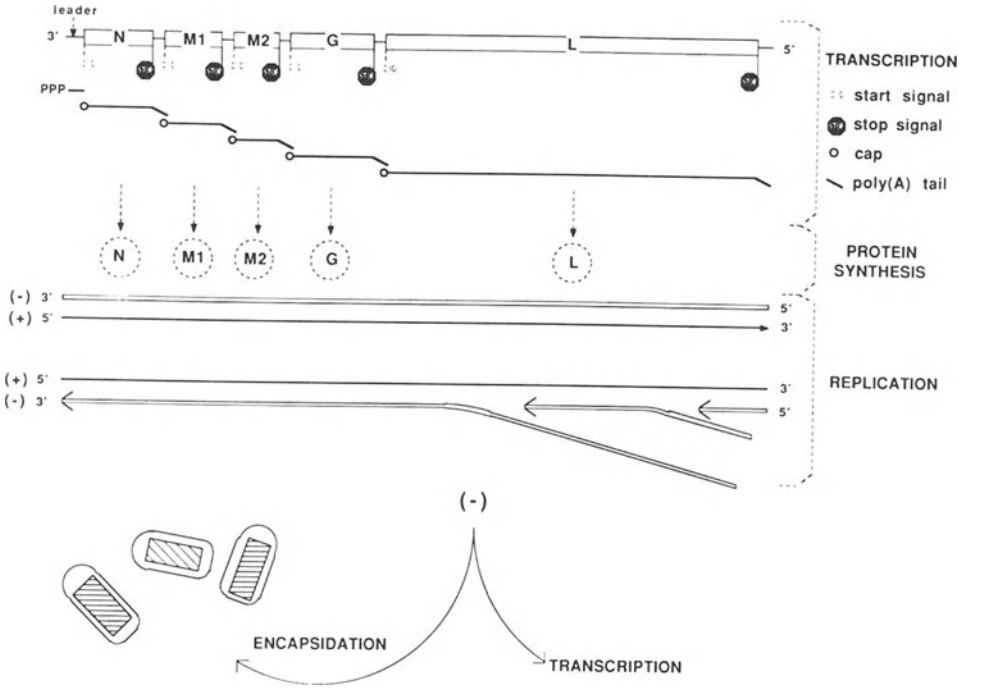

Figure 2. Transcriptional and replicative mechanisms of the rabies genome.

It is only after translation of the monocistronic messengers into rabies proteins that the replication step may begin. This first leads to the synthesis of a full length positive sense genome, an intermediate which is copied in turn to amplify the number of full length negative strand genomes that will either be encapsidated into progeny virions or serve as templates for further transcription.

Implications of Transcription and Replication

The transcriptional-replicative mechanism, shared by all unsegmented negative strand RNA viruses since it allows them to use effectively their non-infectious RNA genomes, has been extensively studied with various viruses and especially with VSV (for reviews see refs. 2 and 54). It predicts some crucial features on which it is interesting to focus attention.

First of all, the fact that the same genomic RNA template is being alternately used for transcription and replication implies that genomic signals must exist to distinguish the limits of the mono-

cistronic transcripts. These transcription signals have been characterized in the majority of unsegmented negative strand RNA viruses studied up to now and appear as conserved sequences, around 10 nucleotides in length, that flank each transcriptional unit (14, 55-57). The deduced mRNA 5' start and 3' stop consensus sequences, although always present in the genomic template, must be recognized as signals only by the transcriptional complex and must be ignored by the replicative one. This raises the unresolved problem of the fine regulation of the RNA-dependent RNA polymerase, which is thought to be the enzyme responsible for RNA synthesis in both complexes.

Secondly, it has been shown for VSV that transcription is sequential (58,59) and progressively attenuated in genes encoded from the 3' to 5' side (60,61), as if the transcriptional complex stopped RNA synthesis at each stop signal, paused between cistrons, and reinitiated only partially at the next start site. Considering the identical rate of degradation of all VSV mRNAs (62), this means that the genomic position of 1 cistron directly influences its transcription rate. In this context, it is interesting to note that the major structural proteins are mostly encoded at the 3' genomic side, whereas the 5' genomic half codes for the catalytic RNA-dependent RNA polymerase as if the regulation of protein synthesis could occur, at least partially, at the transcriptional level. Although rabies transcription has not been studied as completely as VSV, several lines of evidence such as the conservation of the same genomic organization (14,49) indicate that a decreasing rate of transcription may proceed in the same way.

STRUCTURE OF THE VIRAL GENES

A. The Leader RNA.

The leader RNA is the first species synthesized during the transcription process and is therefore produced in the highest molar ratio. It may represent a crucial element of the infection mechanism; nevertheless, it has been characterized only recently in rabies-infected cells (50) and very little is known concerning its precise role. In the case of VSV, its involvement is no more clearly defined although much better documented, and some contradictory observations

can be outlined. For example, although the leader RNA has often been implicated in the shut-off of host macromolecular synthesis (9,63) and more particularly in DNA-dependent transcription (64,65), as suggested by its migration to the nucleus soon after the infection (66), there is no correlation between the extent of this inhibition and the quantity of leader RNA in infected cells (67). The inhibitory effect seems mediated both by the secondary structure of the leader RNA itself (68) and by the interaction of small specific nucleotide sequences with protein factors of the DNA-dependent RNA polymerase (69,70). One of these factors could be the La protein since Kurilla and Keene have shown that both rabies (50) and VSV (69,71) leader RNA can be immunoprecipitated by an antiserum against the La protein.

Besides its probable inhibitory effect on host synthesis, the leader RNA is also certainly involved in the multiplication mechanism of the virus itself, since it is encoded at the genomic extremity where crucial events of this mechanism arise. The leader RNA could therefore serve as a decision point in the switch between transcription and replication (72), or it may include important encapsidation signals (15,73) for the contact of the first neosynthesized nucleoproteins.

B. The Nucleoprotein N

This is a 450 amino acid long polypeptide (13) closely associated with the RNA genome. In contrast with the nucleoprotein of VSV, the rabies nucleoprotein is phosphorylated (45-47). The phosphorylation site has recently been located in the serine residue at position 389 (74). Except for the protective effect on the genomic template leading to the formation of the required template both for transcription and replication, little is known concerning the precise involvement of the N protein in the viral replication mechanism. Such an involvement is nevertheless certain since monoclonal antibodies directed against the N or M1 proteins have been found to be efficient in blocking rabies virus multiplication *in vitro* (75). For VSV it has been proposed that the N protein could modulate the balance between transcription and replication by its ability, when present in sufficient concentration, to bind the nascent positive sense RNA and to mask the normal termination site of the leader RNA, resulting in the

synthesis and encapsidation of a full-length positive sense genome (72). Some other models, which also implicate the phosphoprotein, have been proposed to resolve the enigma of the switch between transcription and replication (2,76-78).

The nucleoprotein may also be involved in immunity since the rabies RNP itself has been shown in a recent study to protect animals against a peripheral challenge with infectious virus (79). An earlier study, however, found that it did not protect against an intracerebral challenge (34).

Comparison of the N protein amino acid sequence with all the sequences existing in protein data banks reveals a segmented homology with the N protein of VSV (13). Some stretches of highly conserved amino acids which are also conserved, although less strongly, in the nucleoprotein sequences of various paramyxoviruses (80), could be involved in the direct interaction with the RNA genome.

C. The Phosphoprotein M1

The rabies M1 protein corresponds to the NS protein of VSV. The "M" nomenclature originally referred to a matrix or membranous position; however, this protein has been reassessed to a more internal position (29,36,37). "NS", which by its name implies "Non-Structural", has been suggested as an alternative (74,75), but this is also a misnomer, no more accurate than "M1". Perhaps "P", for "Phosphoprotein", would be a more acceptable name, although it must be noted that the rabies nucleoprotein N is also phosphorylated (45-47).

The 297 amino acid long M1 protein is present in the virion in 2 unequally phosphorylated forms (81). As in the case of the N protein, the involvement of the M1 protein in viral multiplication has been indirectly demonstrated by blocking with monoclonal antibodies (75). However, there is no clear correlation, up to now, between the phosphorylation state of the M1 protein and its role in transcription or replication, although studies have been devoted to this theme (47).

Interestingly, the M1 mRNA of the PV strain of rabies virus exhibits a second open reading frame, possibly encoding a 102 amino acid long basic protein (14). This is of note since the phosphoprotein mRNA of paramyxoviruses is well known to encode a second basic C

protein of undetermined function, and since overlapping phases have consistently been suggested along the phosphoprotein mRNA of VSV (82-84). Nevertheless, the recent sequence determination of the M1 phosphoprotein gene of another rabies virus strain, the AVO1 strain, provides clear evidence that the counterpart of the paramyxoviral C protein does not exist in rabies virus (85). The second open reading frame most likely represents a remnant of this basic protein.

D. The Matrix Protein M2

The 40 amino terminal residues of this 202 amino acid long protein are mainly proline and charged residues (85). Such a characteristic local composition being observable in many matrix proteins of unsegmented negative strand RNA viruses, one might postulate that a similar role is played by all amino terminal segments. This role, studied mainly *in vitro* with VSV, seems to be an inhibitory effect on transcription (86-88) which indicates that the matrix protein, similarly to the nucleoprotein and the phosphoprotein, is a regulatory element of the multiplication mechanism.

The matrix M2 protein, located on the inner side of the lipidic envelope (29,36,37) appears as a sort of intermediate protein able to interact both with the lipid bilayer and the ribonucleoprotein core, as has been shown for VSV (89,90). This double interaction is associated with the ability of the matrix protein to inhibit the transcription process and to induce a condensation of the RNP (91) and a decreasing mobility of cell membrane-inserted glycoproteins (92), 2 very important steps of the maturation process preceeding the budding of the virion out of the infected cell. Interestingly, analysis of the amino acid sequence has revealed a 19 residue central segment so hydrophobic that it has been predicted by computer analysis (93) to have a high probability of being membrane-bound (14). This is a striking example illustrating how DNA sequencing studies can make an important contribution to the understanding of viral protein structure.

E. The Glycoprotein

The transmembrane glycoprotein G is of crucial importance since it is responsible for the induction and binding of virus-neutralizing antibodies (34) as well as for the stimulation of T cells (94), 2

properties leading to the establishment of humoral and cell-mediated immunity against viral reinfection *in vivo*. The specific regions of the glycoprotein involved in humoral and in cellular responses have been localized (95-100). Furthermore, the G protein mediates the attachment of the virus to the host cells (101). Both its protective role and its function in virus-host cell interactions are considered extensively in following chapters of the present book.

By reason of its importance in vaccination, the glycoprotein gene is the best studied of the rabies genes, and its nucleotide sequence has been determined in 3 different rabies strains: ERA (102,103), CVS (104) and PV(14). The gene product in each strain contains 2 hydrophobic segments typical to its transmembrane character. The first consists of the initial 19 amino acids of the amino terminus. It serves as a signal peptide for the transport of nascent protein through the rough endoplasmic reticulum membrane, but is cleaved from the mature glycoprotein which has a length of only 505 amino acids (105). The second segment, 22 amino acids long, is located in the carboxy terminal part (position 440 to 461) and is thought to be transmembranal. It separates the cytoplasmic hydrophilic carboxy terminal domain (44 amino acids long) from the external glycosylated amino extremity (439 amino acids long). Potential carbohydrate acceptor sites (Asn-X-Ser and Asn-X-Thr) appear in the external glycosylated part of the protein. Two are shared by the glycoprotein of all strains (positions 37 and 319) and additional sites are strain-specific such as a third site in position 204 for CVS, 247 for ERA and PV which also exhibits a fourth site in position 158.

Investigations to determine how the potential carbohydrate acceptor sites are used in the virus (106,107) have revealed that position 319 is glycosylated in all rabies strains studied while position 37 is never glycosylated. The situation is less simple for other positions, in particular position 204 in the CVS strain, which is not always glycosylated, leading to the observation of 2 glycoprotein species, GI and GII, differing in the extent of their glycosylation (43,81). Precise studies with mutants of the CVS strain suggest that the attachment of carbohydrates in position 204 directly depends on the protein folding which determines the accessibility of the site (107).

F. The RNA-dependent RNA Polymerase

This is a giant protein of 2142 amino acids encoded at the 5' side of the genome, occupying 54% of the genomic length (15), and possessing most of the enzymatic activities necessary for transcription and replication (reviewed in ref. 2). This multifunctional role is compatible both with its large size and its presence in catalytical amounts in the virion (42). Furthermore, one example of intracistronic complementation for the L protein of VSV indicates that independent functional sites can arise along the protein. The hydropathicity profile of the L protein is more uniform than those of the M1, M2 and G proteins and no significant hydrophilic or hydrophobic region emerges (15).

A striking feature of the rabies L protein is the very high conservation rate that it exhibits with the isofunctional L protein of VSV (108). One-third of the amino acids of the 2 RNA-dependent RNA polymerases exist in identical positions, a value significantly higher than with other isofunctional proteins, even the nucleoproteins (13,80). The conservation is not randomly distributed, however, and some particular stretches show 75% of strict identity and even 85% if conservative changes of amino acids are taken into account. Still more strikingly, using these high points of homology, it has also been possible to find corresponding regions in the L protein of various paramyxoviruses (15,109,110). This gives the first direct evidence based on substantial sequence data that the Paramyxoviridae and Rhabdoviridae families have emerged from a common ancestor.

G. Genomic Signals

Start and Stop Transcription Signals

Studies of the non-protein coding regions of the genome, and S1 nuclease protection experiments have revealed that all rabies genes are bordered by very conserved sequences that are probably recognized as start and stop signals during the transcription process (14). The resulting mRNA 5' start and 3' stop consensus sequences are presented in Fig. 3. The 2 consensus sequences are closely related to those of VSV (55), sharing 5 identical positions within the 5' start signal and the last 9 nucleotides of the 3' stop signal. At the level of this stop signal, the 7 terminal U residues are thought to be a polyadenyl-

ation site that the RNA-dependent RNA polymerase is able to copy in a reiterative manner, producing the polyadenylation tail of each mRNA before reinitiating at the next start site (111). This polyadenylation step could explain the observation that, in VSV, the transcription process seems to mark a pause between adjacent cistrons (61).

Conserved Extreme Sequences

The 11 terminal nucleotides at both 3' and 5' ends of the rabies genome are inversely complementary. This is classically observed in unsegmented negative strand genomes (112,113). It is highly unlikely that this terminal complementarity could confer to the RNA genome a stable panhandle structure at any moment of the viral cycle, since positive and negative strand genomes are always found encapsidated as soon as synthesized. The stability of the terminal sequences most likely reflects the conservation of important signals, particularly in regions in which crucial biological events occur, such as the initial RNA template-polymerase binding and the initiation of RNA synthesis or encapsidation. Since, for VSV, the polymerase binding site is located in a more internal area of the template, between nucleotides 15 and 35 (114,115), it is more probable that the first 11 represent either a start signal of RNA synthesis recognized by the transcription and replication complexes, or an encapsidation signal for initial contact with the first synthesized nucleoproteins, or both.

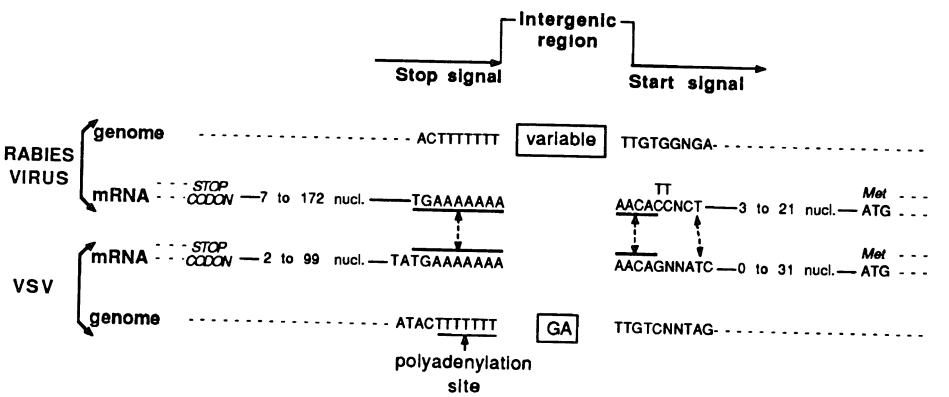

Figure 3. Transcription signals. The 5' start and 3' stop consensus sequences of rabies mRNAs are compared with those of VSV.

EVOLUTIONARY POSITION OF RABIES VIRUS

A. Evolution of Rabies Strains

Since 1981, many nucleotide sequences of rabies genes of various strains have been published. The only genomic RNA sequenced in its entirety is that of the PV strain (13-15), although the genome of an avirulent mutant (AV01) of the CVS strain has been sequenced up to position 3386, including the leader RNA, the nucleoprotein, the phosphoprotein and the matrix protein genes (85). Some authors have taken the approach of determining the protein structure from mRNA cloning and sequencing, although genomic intergenic sequences potentially important in the regulation of the transcripts are not identified by this technique. This is the case for the glycoprotein of ERA and CVS (102-104), as well as for the matrix protein of ERA (116).

A comparison of all the sequence data reveals that rabies strains are highly related, showing between 90% and 98% of amino acid identity (85). The most variable protein is the glycoprotein G, as would be expected of the major viral antigen. Even so, the majority of mutated amino acids are located in the hydrophobic signal and transmembrane segments or in the hydrophilic cytoplasmic domain, and the changes respect the characteristic of each region.

It is curious to observe that the CVS strain appears clearly distinct from the PV and the ERA strains which are very closely related. This fact, evident by comparison of the N, M1, M2 as well as the G amino acid sequences is very surprising since both PV and CVS strains are derived from the French Pasteur isolate (7) whereas the ERA strain was isolated separately in the USA (8). A possible explanation could be that the CVS strain differs from the 2 others by its particular adaptation to mouse brain.

B. Insight into Rhabdoviral Evolution by the Rabies G-L Intergene

Intergenic regions are defined as existing between the 3' stop sequence of 1 messenger and the 5' start of the following one. Unsegmented negative strand RNA genomes can be separated into 2 distinct groups: those with constant intergenes such as VSV (55) and Sendai virus (56) that exhibit the dinucleotide GA and the trinucleotide GAA respectively; and those such as the paramyxovirus respirat-

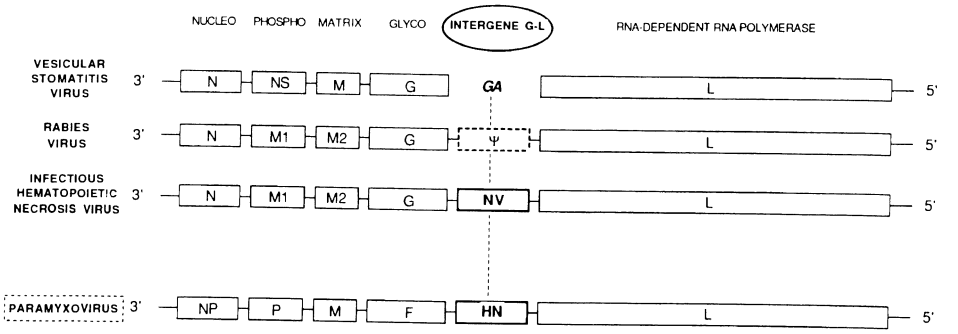

Figure 4. Evolution of the G-L intergenic region through unsegmented negative strand RNA genomes.

ory syncytial virus (57) or rabies virus (14), which show highly variable intergenic regions. The rabies intergenes vary both in length and nucleotide composition. The region separating the G and L cistrons, with a length of 423 nucleotides, is particularly remarkable. The existence of 2 sequences related to the rabies mRNA start and stop consensus sequences near its extremities raises the possibility that it might represent a remnant protein gene. This hypothesis is supported by the identification of a sixth protein gene called NV, similar in length to the rabies G-L intergene, that is encoded between the G and L cistrons of a fish rhabdovirus, the infectious hematopoietic necrosis virus (IHNV) (117,118). The presence of the G-L pseudogene suggests that rabies virus represents an intermediate stage in the evolution of the Rhabdoviridae, located between VSV in which the G-L intergene is next to the dinucleotide GA, and the IHNV which encodes the NV protein at this position (Fig. 4). By extension, it is interesting to note that the additional glycoprotein (hemagglutinin) produced by most of the paramyxoviruses is encoded in a genomic region equivalent to the G-L intergene. This emphasizes the plasticity of this region in the unsegmented negative strand RNA viruses.

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3

CULTURE OF RABIES VIRUS *IN VITRO*

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ABSTRACT

Since the early 1950s, cell culture systems have been developed which have led to the understanding of much of the structure and biology of rabies virus and have also made it possible to grow the virus in sufficient quantities for vaccine production. In this chapter, we give a brief historical account of the production of the wide variety of cell systems available today and of how they have been employed in the various areas of research and virus assay. The information gained in terms of virus infection and pathogenesis *in vivo* is discussed, as are the ways in which cell cultures are now applied in the important areas of diagnosis and epizootiology of the disease. There is a short résumé on vaccine production and the problems of transferring the appropriate technology to the developing countries. We conclude by considering the many questions that remain and how tissue culture techniques may assist in providing some of the answers.

INTRODUCTION

Rabies virus (RV) is remarkable in its apparent ability to infect and kill all mammalian species. The virus is regarded as highly neurotropic, yet in the infected animal after replication in the central nervous system it spreads centrifugally to most organs in the body, in which it is often able to replicate efficiently. It is not surprising, therefore, that *in vitro* rabies virus can grow in a wide variety of cells (see reviews 1-3). Indeed, much of the information contained in this book could not have been obtained had cell culture techniques been unavailable.

HISTORY

Attempts to grow RV *in vitro* date from before World War I when Noguchi (4) and Levaditi (5,6) independently reported the prolonged release of virus from cultured fragments of nervous tissue taken from infected animals. No further attempts to culture the virus were reported until after 1930 when it was shown that fixed RV would replicate in both mouse and rat embryo brain cultures and tumor cells (cited in refs. 2,3).

The susceptibility of primary mouse kidney cell cultures to RV infection was reported by Vieuchange and coworkers in 1956 (7,8). Two years later, Kissling described the successful serial passage of both street and fixed virus in primary hamster kidney cells (9), and by 1963 Kissling and Reese (10) had demonstrated the potential use of virus grown this way for the preparation of a vaccine. It has since been shown that large scale production of RV is also possible in a variety of primary culture systems including monkey (11), dog (12) and pig kidney (13), and chick embryo (CEF) and duck embryo fibroblasts (14).

Nowadays, there are a number of cell lines and cell strains available for the regular production of large quantities of RV (2,3). Such cell lines include BHK-21 (15), Nil-2 (16), CER (17) and Vero (18). Neuroblastoma cell lines of mouse (19-22) and human (3,21) origin are very susceptible to RV infection, and are frequently used in diagnostic tests and for the study of virus virulence (19-22).

Because of their heteroploid characteristics and oncogenic potential, none of the cell lines, with the exception of Vero cells (18,23), has been considered suitable for the production of human vaccines. However, in 1964 Wiktor and coworkers (24) described the production of vaccine in a human diploid cell strain, WI-38. Other human diploid cell strains such as HEL (25) and MRC5 (26) have been used for the same purpose, and a rhesus monkey diploid cell line vaccine has also been produced (27,28).

The propagation of RV has also been demonstrated in a number of cell lines of poikilothermic origin (2,3) but not in insect cells (29). Other unusual cells, e.g. embryonic chick myotubes (30) and a mouse macrophage cell line (31) have also been used for *in vitro* studies of RV infection and pathogenesis.

Far less information is available on the ability of the rabies-related viruses (see King and Crick, this volume) to grow in cell cultures. Lagos bat (3), Mokola (3) and Duvenhage (32) viruses will grow in BHK-21, CER, Vero and C1300 neuroblastoma cells (3,17,32). Obodhiang and kotonkan viruses are cytopathic for neuroblastoma cells (3) and also grow in cells of Singh's *Aedes albopictus* mosquito cell line, after which they can be passaged in BHK-21 and Vero cells (29,33). Interestingly, Mokola virus differs from the other 2 human pathogens, RV and Duvenhage virus, in that it can replicate in both vertebrate and invertebrate cell cultures (33).

VIRUS PRODUCTION

Propagation

Although a wide variety of cell types can be used to propagate RVs, many systems require considerable periods of adaptation and prolonged passaging before substantial virus yields can be obtained (1-3). In this respect, diploid cells are particularly difficult as exemplified by the original adaptation of the Pitman-Moore strain to WI-38 cells (1-3,24). Prolonged and regular serial passage of infected cells, initially with the addition of fresh uninfected cells, was required before sufficient infectious virus was released into the culture supernatant fluid. Only at this stage could these fluids be held as virus stocks and used for subsequent reinfection of fresh uninfected cells. The yield in WI-38 cells, even with adapted virus, is only about one tenth of that when cell lines such as BHK-21 or Vero are used - yet another reason for restricting the use of diploid cells to the production of vaccine for humans.

However, regardless of the type of cell being used, there are a number of factors which can have a profound effect on virus production. These include the pH of the culture medium, the presence or absence of growth supplements (such as serum or serum albumin) and temperature control (1-3,34). Optimum conditions seem to vary between laboratories, the "sub-strain" of cells used, and in the case of cell lines such as BHK-21, local differences in the composition of Eagle's MEM, the most commonly used medium (2,3). The quality of cells at the time of initial infection is critical, and in order to obtain maximum yield of virus it

is vital to use cultures which are just confluent, having been passaged not more than 2-3 days previously. High input multiplicities of infection (MOI) may occasionally lead to autointerference (35-37), though this is not normally a problem. Nevertheless, efforts to produce single cycle growth curves in RV-infected cells have, in general, been unsuccessful. This may be related to the fact that replication proceeds relatively slowly and that it is not always possible to produce synchronous cell populations (2,3).

Cytopathology

In general, no specific cytopathic effect (CPE) accompanies the production of RV in tissue culture. In infected BHK-21 cell monolayers, for example, the cells merely begin to "age" and detach more quickly from the supporting surface than uninfected control cells. Again, there are exceptions to the rule. In monolayers of chick embryo fibroblasts, Yoshino and coworkers (35-37) found that the viability of infected cells was sufficiently affected for plaques to develop. Similarly, plaques could be induced in infected agarose-suspended BHK-21 cells (38), and plaque-forming viruses could also be recovered from persistently infected cells of the same type (39).

In neuroblastoma cells with RV, Lagos bat, Mokola and Duvenhage viruses, infection is much more severe than in BHK-21 cells. Duvenhage actually induces the formation of syncytia (22). Obodhiang and kotonkan viruses will form plaques in these cells (22).

Persistent Infection

Since the first reported isolation of RV defective interfering particles (DIs) (40), their production by many virus strains in many cell systems has been generally recognized (3,41,42). They are in fact extremely readily produced: for example, detectable levels of DIs can be generated in the first undiluted passage of a cloned pool of HEP Flury virus (41). Purified rabies DIs, like those of other rhabdoviruses, interfere in culture with the production of homologous and closely-related strains (40,43,44; King and Crick, this volume), and are probably involved in the establishment of persistent *in vitro* infections, a subject discussed in some depth by Holland and coworkers (43,44). Thus, without their initial introduction, DIs are produced during the establishment of persistently infected cultures, and

alternating cyclical production of infectious virus is observed. An earlier conclusion that this pattern of virus production, which was accompanied by the production of interferon or an interferon-like substance (2,45) is not necessarily contra-indicated by these more recent observations.

Serial propagation of viruses can also affect phenotype (46). Andzhaparidze and coworkers (47) reported that, as in the case of many other virus infections, the host cell type persistently infected influenced both the virus DI interactions and the virulence phenotype of the released virus. Wunner and Clark (48), however, working with both virulent and avirulent viruses, were unable to find any correlation between DI production and virulence phenotype.

Whatever mechanisms are involved in chronic or persistent infection, RV has a clearly established endosymbiotic relationship with the host cells, which are able to continue growing and replicating as efficiently as control uninfected cells (2,49). Considerable effort to explain persistence in molecular terms has been made in many laboratories, and the reader is referred to a comprehensive review on the topic by Wunner (50).

Virus in Infected Cells

In 1903 Negri and Bosc independently described inclusion bodies associated with rabies infection of cells of the brain, their presence or absence subsequently providing a practical method for establishing diagnosis (51). It was not until much later that "Negri bodies" were shown to contain viral antigen (52) and viral particles within the brain cell inclusions were demonstrated by Matsumoto (53,54). Using techniques similar to those of Matsumoto, coupled with the use of ferritin-labelled antiserum, Hummeler and coworkers demonstrated that the similar inclusion bodies in BHK-21 cells, although containing virus particles, were largely composed of ribonucleoprotein (55).

A number of other methods for detecting virus infection *in vitro* are now available. For convenience in diagnostic tests, conventional staining has been largely replaced by the immunofluorescent antibody technique (IFA), a subject reviewed in detail by Kissling (56; see also Webster and Casey, this volume). The IFA technique has also become the method of choice for following the progress of infection in tissue

culture. With fixed cells, the antigen predominantly stained is the N protein of the nucleocapsid (see Tordo and Poch, this volume), whereas staining of unfixed cells reveals mainly viral glycoprotein (G) located on cell plasma membrane (3).

Routinely, polyclonal hyperimmune antisera are still employed in the IFA test, but in the last 10 years monoclonal antibodies directed mainly against the N protein (Mab-Ns) or the G protein (Mab-Gs) have become available (57). These provide more specific probes for distinguishing between cells infected with RV or other members of the genus *Lyssavirus* (see King and Crick, this volume). Usually, the cells are treated with the appropriate unlabelled Mabs whose coupling with the viral antigen is then revealed by the addition of a labelled anti-mouse antiserum (58).

Although the IFA test is the most reliable and sensitive indicator of RV infection, it does not necessarily provide a measure of infectious virus within the cells nor of their potential to release virus into the culture medium, a feature to be borne in mind when using the test to monitor virus production, e.g. for vaccine manufacture.

Compared with IFA, other tests for the detection and examination of infected cells are comparatively little used. However, some tests have particular value for specific circumstances. Complement-dependent immune lysis may be useful for detecting cells which are beginning to release virus or from which there is only a low level of virus release as in chronic infections (59), although reservations about use of the method for this purpose have been expressed (60). Electron microscopy has also been employed to examine the course of infection (e.g., 50, 55,61).

APPLICATION OF TISSUE CULTURE METHODS

Rabies Virus Assay

Somewhat surprisingly in view of the limited ability of the viruses to induce CPE, a number of plaque assay methods have been developed. Cell lines used for this purpose include BHK-21 (both the C13S sub-strain maintained in suspended culture (62) and BHK-21/C13 maintained in monolayers (63)), CER (64), the pig kidney cell line PK-2A (65) and Vero (66). Chick embryo fibroblasts have also been employed

(35-37). The most widely-used technique is based on agarose-suspended BHK-21/C13S cells in which most culture-adapted fixed RV produce plaques after 5-7 days of incubation (38,62).

The plaques obtained in CEF by Yoshino and coworkers (36,37) were regarded more as proliferative foci than the type of dead cells of which plaques are usually composed. More recently, Kawai and Matsumoto (68) developed an interference focus-forming technique in BHK-21 cells for the assay of DIs. The same workers have also shown that virus development is influenced by the host cell (67).

Lagos bat and Mokola viruses can also be assayed by plaquing in BHK-21/C13S cells (3), Mokola virus in a pig kidney cell line (69) and the entire lyssavirus group in Vero cells (66). Obodhiang and kotonkan can be titrated by the plaque method and by CPE in C1300 neuroblastoma cells (22).

Unfortunately, plaque assays do not always give consistent results even with well-adapted fixed virus strains. For virus titration, they have been largely superseded by fluorescent focus assays in which cells, e.g. CEF or BHK-21 (70) are incubated with serial dilutions of virus, then fixed and treated with fluorescent serum or Mab-Ns 1-4 days later. Titration endpoints compare well with those obtained by intracerebral inoculation of mice (70). The test has proved suitable for fixed and street strains, not only of RV but also of Lagos bat, Mokola and Duvenhage viruses (see King and Crick, this volume)

Virus neutralizing antibody can be titrated by either plaque reduction methods (71) or, more conveniently, by fluorescent focus inhibition tests (70; Campbell and Barton, this volume). Results with these tests also correlate well with those obtained by the mouse neutralization test. As an extension of the methods, both have been used in antibody-binding tests whereby vaccine potencies may be determined (72).

Infected culture fluids also include non-infectious virus particles and sub-viral components. Complement fixation tests can be used to measure total viral antigen (73) and hemagglutination tests using goose erythrocytes to measure intact particles (74). This method requires a concentration of at least 10^6 PFU/ml, and can only be used for virus assay in serum-free medium (3).

Virus Structure and Strategy for Replication

Our knowledge of RV structure would be extremely limited without the ability to grow and radiolabel virus in tissue culture prior to its purification and analysis by the techniques of molecular biology. In addition, cells in culture can be used to study the processes of virus infection, transcription and translation, replication, assembly and release. In addition, many of the effects of virus on cellular metabolism can now be examined at the biochemical level; for details, the reader is referred to the major review articles in "The Rhabdoviruses" (75), and the chapters in this volume by Tordo and Poch, and by Tsiang.

Pathogenesis

Despite more than a century of continuous effort in laboratories throughout the world, our understanding of, and therefore ability to intervene in, the course of rabies infection remains extremely limited. Current concepts of rabies pathogenesis have been reviewed by Wunner (50) and by Charlton (this volume). Our remarks will be confined to the contribution tissue culture techniques have made, and can make, in its elucidation.

Many of the earlier experiments employed thin section electron microscopy to visualize how infection occurs, the fate of the virus once it has entered the cell, morphogenesis, and release of nascent virus. However, with the exception of Matsumoto and coworkers (cited in 50) where *in vitro* and *in vivo* studies were done in parallel, most of these experiments were made in BHK-21 or CER cells, despite the fact that in the intact animal replication may be almost entirely restricted to nervous tissue. Results appear to vary with experimental conditions, i.e., the strains of virus, MOI and type of cell employed (67,76).

The observations of Iwasaki and Minamoto with chronically infected C1300 neuroblastoma cells examined by the newer technique of scanning and freeze fracture electron microscopy may have more bearing on what happens in natural infection (77).

Major developments in the study of pathogenesis have resulted from the availability of the plaque technique and thus the possibility of selecting virus "clones" and mutants. An extension of this method whereby a strain of virus is grown in the presence of suitable neutralizing Mab-Gs has made it possible to select variants (78,79) with

altered pathogenic (78) or protective (80) potential whose antigenic changes can be subsequently mapped (78-80).

As an adjunct to these studies, the Wistar group have compared the cell-to-cell spread of pathogenic parental virus and apathogenic variants *in vivo* (in the brains of infected adult mice) and *in vitro* (in BHK-21 and neuroblastoma cells). Cultured neuroblastoma cells retain some characteristics of neurons, and Dietzschold and coworkers (81) were able to correlate differences in pathogenic behavior between the 2 types of virus in both *in vivo* and *in vitro* systems. Interestingly, no differences between pathogenic and apathogenic virus infections were observed in BHK-21 cells (81), a factor underlying the importance of selecting the appropriate model for each experimental situation.

Suitable virus receptors are necessary for successful infection and the host range of many viruses may therefore be determined initially by their presence or absence on the cell surface. A specific receptor for RV has not yet been identified, if indeed one exists, although the involvement in infection of both muscle spindles and motor endplates in striated muscle has been implicated (82).

Several groups of workers have begun to search for receptors in cultured cells. Prominent among these are the group at the Pasteur Institute (see Tsiang, this volume). Some experiments have been conducted in conventional systems such as CER and neuroblastoma cells (83,84) but a highly sophisticated compartmentalized technique for the culture of dorsal root ganglion cells has also been developed (85). The system allows the infection and manipulation of neuronal extensions without exposing the neural soma to the infecting virus. Thus the high binding affinity of the virus to unmyelinated neurites and its transfer by the neurites to the neuronal soma has been shown, thereby supporting the view that sensory nerves can indeed be involved in the centripetal transfer of virus to the central nervous system (CNS).

In contrast, a group of workers at Yale have sought to substantiate the observation made in their laboratory that motor endplates and hence motor nerves could be of prime importance in the transfer of virus to the CNS following its initial introduction to the body (86). A series of experiments involving first, isolated mouse diaphragm with

attached phrenic nerves, and then cultured embryonic chick myotubes (both chosen for their high density acetylcholine receptors (AChR)), led them to the speculation that this receptor might be involved in RV infection: at the motor nerve terminal region post-synaptic AChRs are abundant (30,87). However, tests of this hypothesis in a number of cell culture systems including some lacking high density AChRs have led Reagan and Wunner (88) and Tsiang (89) to the independent conclusions that AChRs are not necessary for RV infection, and that the susceptibility of different cell types does not depend on a single specific type of receptor (50,88,89; Tsiang, this volume).

There is, however, increasing evidence that lipids (83) and carbohydrates (90) may be involved in the early interactions between RV and cell membranes. Again, the availability of suitable cultured cells is crucial in the investigations, other references to which are given by Wunner (50).

Comparatively little attention has been paid by most investigators to the possibility of an immunological involvement in at least some cases of RV infection, e.g., the "early death" phenomenon (91-94). However, evidence for antibody involvement has now been presented by a number of authors (referenced in Wunner's review, 50). In a test using the P388DI mouse macrophage cell line (95), King and coworkers (31) were able to show that rabies antiserum diluted beyond the neutralization endpoint enhanced the ability of virus to infect these cells. A similar effect in the intact animal could go some way to explaining why certain animals or patients succumb to the disease despite vaccination and indeed die more rapidly than infected but unvaccinated individuals.

Diagnosis

Even in countries where rabies is not endemic, a diagnostic capability which includes virus isolation and identification is desirable, and for many years a replacement for the mouse test has been sought. Smith and coworkers (17,96), using CER and neuroblastoma cells, were the first to explore the potential of culture systems for this purpose.

The system of cell culture has since been adopted in some laboratories (97-99), in many of which BHK-21 cells have not been regarded as sufficiently sensitive for use with field isolates. However, at

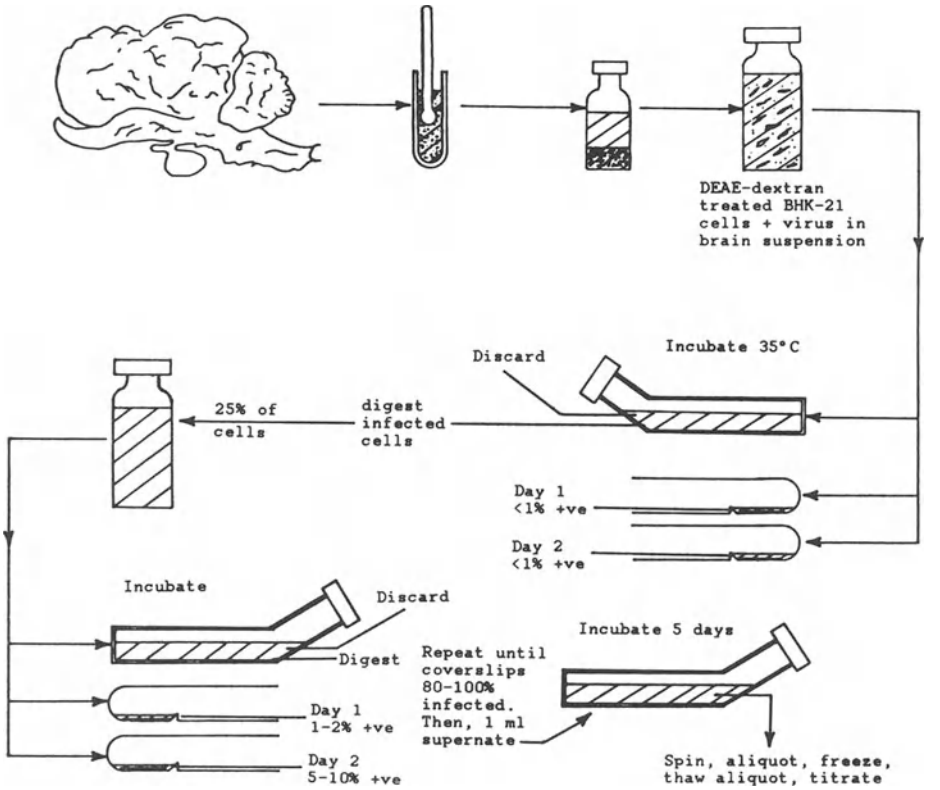

Figure 1. Procedure for isolation of RV and rabies-related viruses from field specimens using cell culture techniques.

Weybridge, we have found these cells satisfactory for diagnosis and the preparation of virus stocks.

Central to our method is the maintenance of cultures, and as in the case of virus production for other purposes, including vaccines, the quality of the cells used is an all-important factor. Monolayer stock bottles are passaged every 3 or 4 days using a 1:4 split ratio, and cells for infection are never used at more than 3 days of age. BHK-21 cells maintained in this fashion are hardy, requiring a relatively simple medium supplemented by inexpensive ox serum, and since they do not require additional CO_2 to support growth, they have a possible safety advantage over open culture systems. Culture bottles surplus to immediate requirements are stored at ambient temperature or at +4°C, and passage levels are kept low by returning to these cells up to 2-3

weeks later, when after 1 or 2 passages they regain their rapid growth characteristics.

Cells in exponential growth phase are far more susceptible to RV infection than those in lag or decline phase, and the addition of DEAE-dextran to a final concentration of 100 µg/ml to suspended cells shortly before infection further increases their susceptibility (1,2,100).

To determine parameters for the virus isolation method (Fig. 1), 1 ml of a 10% suspension of an infected fox brain was mixed with 80 ml suspended BHK-21 cells, and from the mixture 6 25 cm^2 bottles and 12 Leighton tubes were seeded. The following day, a coverslip was examined, an estimate of the percentage of infected cells made, and a sample of supernatant was stored at -70°C. The cells in the bottle were trypsinized, and a portion (usually 25% but more if the brain material had had a deleterious effect on the growth rate) passaged. Other samples were similarly treated on day 2, or on day 3, 4, 5 or 6. When the cells in the Leighton tubes indicated that more than 50% of the cells in the bottles were likely to be infected, these cells were trypsinized and seeded into an 80 cm^2 bottle which was incubated for 6 days, during which it was sampled daily. At the end of the experiment, all samples were titrated in one test, and after 4 days' incubation were fixed, stained, and the titers recorded.

Results indicated that rapid passage of infected cells led to early adaptation of the virus to BHK-21 cells (A. King and P.K. Davies, unpublished data), with the advantage of limiting the opportunity for change in phenotype between the field isolate and laboratory stocks (22,46). Indeed, the highest titers were obtained when cells were passaged either daily or at 2 day intervals, and by 9-12 days virus stocks suitable for Mabs studies were obtained.

By comparison, of five 21-day-old mice inoculated intracerebrally with the 10% brain suspension, none died of RV infection before the 13th day.

The method has been successfully used for the isolation of RV and rabies-related viruses from brain material from many species and from many parts of the world. It was also used for the isolation of RV from 5 of 6 saliva samples taken on consecutive days from a patient who

Table 1. Tissue culture rabies vaccines currently manufactured

Substrate	Inactivated	Modified live	Application	
			Human	Animal
Primary cells				
hamster kidney	+	+	+	+
dog kidney	-	+	-	+
fetal calf kidney	+	-	+	-
pig kidney	-	+	-	+
chick embryo fibroblasts	+	+	+	+
Diploid cell strains				
human lung - WI-38	+	-	+	-
human lung - MRC5	+	-	+	-
rhesus monkey lung - FRhL-2	+	-	+	-
Heteroploid cell lines				
monkey kidney - Vero	+	-	+	+
hamster kidney - BHK-21	+	+	-	+
hamster kidney - Nil-2	+	-	-	+
dog kidney	-	+	-	+
pig kidney	+	+	-	+

Sources of information: refs. 23,24,26-28,101-103. This is not intended as a comprehensive list: it merely shows examples of the wide selection available.

later died of rabies contracted abroad (A. King and P.K. Davies, unpublished data).

Vaccines

Effective vaccination policies are so much part of the prevention and control of rabies that probably the most important use of tissue culture is in the production of vaccines. Cultured vaccines are free from many of the side effects of nerve tissue and avian tissue vaccines, store well, and are highly antigenic. They can be prepared in primary cell cultures, in cell lines and in cell strains, and may consist of modified live (attenuated) or inactivated viruses. An idea

of the enormous variety of vaccines manufactured throughout the world can be gained from references 92,101-103 and Table 1 (see also Bunn, this volume). Cells such as BHK-21 and Nil-2 are particularly good sources of antigen, and can be grown in bulk in suspension cultures (34) or as monolayers or on microcarriers (104).

With the increasing availability of highly potent inactivated vaccines, the use of live vaccines for domestic animals is being discouraged, especially where rabies has been eliminated or is under control (92). They are, however, being distributed in bait for the control of wildlife (fox) rabies in Europe and Canada (105,106; see also chapters by Wandeler and MacInnes, this volume).

Live vaccines are not used for humans, and for medical use the choice of cell substrate has until recently been confined to primary cell cultures (102) or human cell lines such as WI-38 (24) or MRC5 (26). Because of technical difficulties and the consequent high costs of these products, alternatives have been sought and vaccines can now be produced in a rhesus monkey diploid cell line (27,28). It now appears that Vero cells are also acceptable as a substrate for human vaccine manufacture. These cells have the advantage of being readily adapted to growth on microcarriers (18,23).

There are, however, certain disadvantages in maintaining infected cells for vaccine production in large scale propagators, not least of which is the need to supplement the medium with serum or serum substitutes (23,104). This does not apply if roller bottles are used, and a very low medium volume: cell ratio can be maintained. The method, though more labor intensive than with tanks, is simpler, the equipment easier to install and maintain and, in the event of contamination of one roller, the entire batch need not be discarded. These are factors which should not be ignored when considering the feasibility of transferring vaccine production to developing countries (107).

Killed vaccines are often concentrated, and, for human use, purified before inactivation (23,102,103). Their potency can then be monitored by antibody binding tests (102) which can be conducted in the same type of cell as is used for production purposes (71,72,102). Blind passages of the product in uninfected cells are often made to confirm the efficacy of virus inactivation (108).

DISCUSSION

Anyone who has ever worked in a virus laboratory, whether in research, diagnosis, or vaccine production knows only too well the frustration and gloom when there are "no cells". In this situation, in order to justify one's existence it becomes necessary to visit the library "to catch up on the literature" or worse - "to start to write the long overdue paper". Yet this very predicament is a measure of the reliance we place on tissue culture techniques and their vital role in so many of our laboratory activities.

We have attempted to discuss the many aspects in which cell culture has so far provided a knowledge of RV structure, pathogenicity and assay techniques, and been of immense value in disease control. Much remains to be done. Undoubtedly, even more sophisticated methods will soon provide the appropriate procedures to further understanding of, for example, the infection process and the cellular responses thereto.

No attempt has been made to consider the early results obtained using inhibitors to probe the course of infection and explore the role (if any) of the cell nucleus in virus replication, or of the ability of RV to superinfect cells already carrying other viruses, all of which have been discussed by Wiktor and Clark (2). Space has not permitted us to consider the production of interferon by RV and some rabies vaccines, nor of the sensitivity of the virus itself to interferon (2,45,50). What does the production of DIs mean? Do they have a role in persistent infection *in vivo*, and what pertinent information can be gained by future work with persistently infected cells? Could further studies be directed towards the possibility of latent or persistent infection in bats?

A start has been made in the examination of host cell specificities and virus receptors (see above and Wunner (50)), and Koschel and Munzel (109), using a rabies-infected hybrid cell line (mouse neuroblastoma x rat glioma), have shown that normal cellular functions can be modified *in vitro* by the infection. The reverse, i.e., the ability of the host cell to influence the outcome of infection has been shown (e.g., 21,46,67). At a practical level, Blancou and colleagues (110) have shown that it may be possible to attenuate currently circulating

street viruses sufficiently for their use as vaccines. No molecular explanation for any of these observations is yet forthcoming.

The recent recognition that rabies-related viruses are not confined to Africa and that Duvenhage virus is well-established in at least 1 species of European bat (see King and Crick, this volume) has reawakened interest in the entire group of lyssaviruses. However, the host range of serotypes 2-4 and of Obodhiang and kotonkan viruses seems more restricted than that of classical RV (serotype 1). Yet Mokola (serotype 2), Lagos bat (serotype 3) and Duvenhage (serotype 4) viruses seem to grow as easily as RV itself in culture, and even the insect-borne viruses Obodhiang and kotonkan can be adapted to some of these cell systems (3,22,66).

Sufficient Mabs are now available, at least in the developed countries, to make it possible to monitor the emergence not only of rabies-related viruses in unexpected places, but strain differences within RV itself. Such techniques could be important not only in modifying production of conventionally cultured vaccines but in the suitable tailoring of the next generation of genetically engineered vaccines which themselves are at present produced in culture (111; see also Rupprecht and Kieny, this volume).

Variant viruses produced by growing strains of RV in tissue culture in the presence of Mabs have been described (78-80) but it is difficult to imagine similar antibody pressures in the intact animal since the infecting virus spends so much time in immunologically protected sites of the nervous system, and usually kills the host. Recovered or persistently infected animals are relatively rare (112). Do these considerations also apply, for example, to European bats, some of which appear quite healthy despite being infected with Duvenhage virus? (See King and Crick, this volume).

With all these problems and many more to solve, for the foreseeable future rabies workers will continue to need their cell cultures.

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4

INTERACTIONS OF RABIES VIRUS AND HOST CELLS

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INTRODUCTION

Studies of the pathogenesis of rabies involve investigation of the etiological agent as well as the host response to the presence of the pathogen. The morphological and structural similarities to other members of the Rhabdoviridae are striking and vesicular stomatitis virus has been a useful molecular model for understanding replication of rhabdoviruses (see Tordo and Poch, this volume). On the other hand, the host response to rabies infection has unique features in regard to viral tropism, mode of transport in the nervous system, and functional alterations that lead to lethality. Interaction of rabies virus with host cells covers many topics. This chapter deals mainly with the response of cultivated cells to rabies virus infection, and occasionally with the *in vivo* situation.

Development of methods to cultivate viruses *in vitro* markedly broadened the scope of rabies research by providing 2 new avenues of investigation. The first consisted of cultivating rabies virus in cells or fragments of tissue derived from neuronal structures in order to reproduce the natural virus-host interactions. The second was to adapt and cultivate rabies virus in a suitable cellular substrate, to produce large quantities of rabies virions either for manufacturing rabies vaccines or for studies of virus structure. During the past few decades, most efforts have been directed toward the production of large quantities of virus, essentially in fibroblasts, and little concern has been given to studies of pathogenesis. Recently, however, new tools in virology and in neurobiology have renewed interest in using neural cells for studies of virus-neuron interactions and the mechanisms of pathogenesis in natural target cells.

J.B. Campbell, K.M. Charlton (eds.), RABIES.

In this chapter we discuss the mechanisms of virus-cell interaction. These mechanisms include the mode of entry of rabies virus into susceptible cells, and its release into the intracellular compartment, replication, assembly and release into the extracellular environment. The tropism of rabies virus toward muscle cells which might represent the primary target cells under certain conditions, and the direct infection of peripheral neurons are discussed. The subsequent infection of the central nervous system (CNS) that contains the final target cells is also described. Absence of viremia is a specific feature of rabies virus infection; viral transport from the peripheral site of inoculation to the CNS through neuronal pathways and viral spread in the brain are therefore essential steps in the pathogenesis of rabies. There is also increasing evidence that rabies virus-mediated lethality may be the consequence of neuronal function impairment.

RABIES VIRUS TROPISMS

Historical Review

In a short communication, Pasteur *et al.* (106) provided the first demonstration of the presence of rabies virus in the nervous system by inoculating healthy animals with suspensions of brain (cortex, brain stem and spinal cord) from a rabid dog. Pasteur *et al.* (107) also demonstrated the presence of rabies virus in peripheral nerves and were the first to try to cultivate rabies virus in sterile fragments of neural tissues. It was Noguchi (101), however, who reported infection of cerebral and medullar neural tissues maintained *in vitro*. He observed the presence of inclusions which might have been Negri bodies. Levaditi (73,74) reported the replication of rabies virus in fragments of neural tissues derived from experimentally inoculated animals. These infected tissues could be maintained for two months, although most of the cells at the end of that time would have been fibroblasts.

Consecutive passages of rabies virus were successfully achieved using embryonic chicken brain (126). Later, use of rabbit (59,60), mouse and rat (15,63,109,147,148) embryo brains allowed serial passages of rabies virus. Cultures made from embryonic mouse brain were found to be more efficient than those from newborn or adult mouse brain for viral production (14,104,145,146). And for the first time, Plotz and

Reagan (109) succeeded in infecting tissue cultures with viruses isolated from human and canine brain materials without any adaptation step.

These early studies were difficult because neuron culture technology was at its beginning, and the neurons were always contaminated by other cell types. Moreover, only light microscopy and histological stains were available, the electron microscope being introduced later. The specific fluorescent antibody method (38), titration of infectious rabies particles in plaques (120,123,156), the use of neuronal cell lines (150), the techniques of primary neuron cultures (77,139,142) were tools available only in the following decades.

Neurotropisms

Replication of Rabies Virus in Neuronal Cell Lines

Established cell lines of neural origin provide tools for the investigation of rabies pathogenesis *in vitro* (23,30,48,53,65,142). These neuronal cell lines have 2 main advantages: cellular homogeneity and continuous passages. They also possess specific neuronal properties closely similar to those of the normal neurons (2). The comparative susceptibility of cell lines of neuronal and other origins to fixed rabies virus (brain-adapted CVS strain) infection demonstrated that

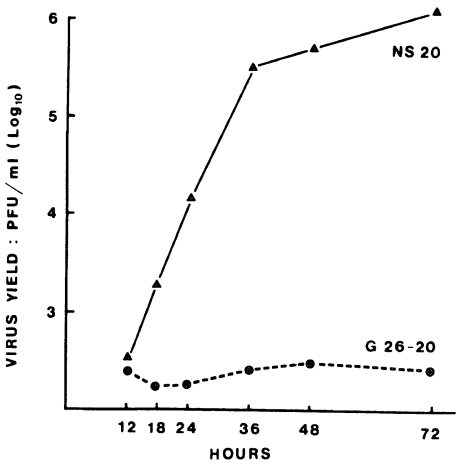

Figure 1. Comparative growth curves of fixed rabies virus in NS 20 neuroblastoma and G 26-20 glial established cell lines. Both cell lines were infected with BHK-adapted CVS (m.o.i. 2 PFU/cell).

only murine neuroblastoma cells produced high yields of infectious virus, whereas glial and BHK cell lines were not productive and CER cells had an intermediary virus production (142). High viral titers are usually obtained in BHK cells after adaptation by serial passage either in the BHK line itself, or after adaptation in other cell types (7). After serial passage, rabies virus can be adapted to grow in many cell systems (149,150), including glial cell lines (5).

The BHK-adapted CVS strain grows to a much higher titer in NS 20 neuroblastoma cells than in G 26-20 glial cells (Fig. 1). The greater permissiveness of neurons with respect to glia is observed at 2 levels: a higher ratio of infected neurons and a higher yield of virus per infected neuron. Different clones of murine neuroblastoma cells which exhibit neuronal properties had identical susceptibility (142). Murine neuroblastoma cells have also been shown to increase the virulence of rabies virus when propagated in these cultures (22), and to support back mutation of attenuated viruses to the virulent state (23). Human neuroblastoma cells also exhibit high susceptibility to fixed rabies as well as to street rabies virus strains (48,49).

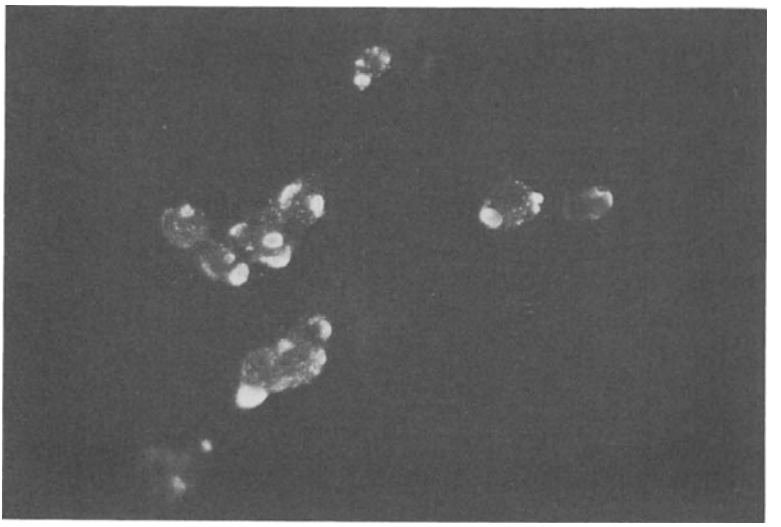

Figure 2. Infection of dissociated DRG neurons by fixed rabies virus. Dissociated neurons from 14 day-old rat embryos were cultivated in collagen-coated Petri dishes and infected with CVS virus (10^5 PFU/ml) (77). After 30 hrs' incubation, the neurons were stained with an anti-rabies nucleocapsid fluorescent conjugate (x 160).

Viral Replication in Organized Cultures and in Dissociated Primary Neurons

Improvements in techniques allowed further investigations of the cytopathic effects of rabies virus on neuron cultures (34) and electron microscopic examination of rabies virions in organized cultures of dorsal root ganglia and spinal cord from fetal rodents (88). The neurons were shown to be sensitive to both fixed and street rabies virus and to show signs of cytopathic effects (CPE) 3 days after infection followed by neurolysis. Some glial cells were also reported to be infected. Infection of neurons in organized cultures by both fixed rabies and street rabies viruses showed an inverse relationship between CPE and the presence of virus. The virus yields in both viral strains were low and did not reflect the kinetics of viral replication (88). In addition, studies with the same type of cultures demonstrated that the site of viral assembly occurred on membranes of the Golgi complex. Virus budding was observed to occur on the cell surface membranes as well as on the intracytoplasmic membranes (87).

Infection of primary neuronal cells with rabies virus without any adaptation step showed that these cell types were particularly susceptible to the viral infection (139,142,144). Dissociated cell cultures of nervous tissue origin can be obtained from mouse or rat embryos (14-18 days) by mechanical or enzymatic treatment. These cultures can be enriched in neurons by cytosine arabinoside treatment (1 µg/ml) 24 or 48 hrs after seeding the cells. Neurons derived from mouse dorsal root ganglia (DRG) and CNS were highly susceptible to fixed rabies virus infection: 24 hrs after infection, more than 90% of the neurons exhibited viral inclusions as monitored by specific immunofluorescence, whereas only 1-10% of non-neural cells were infected (142). In this series of experiments, neurons were easily identified by morphological criteria, and central neurons were stained by tetanus toxin binding. In addition, astrocytes were identified by antibodies to glial fibrillary acidic protein, and oligodendrocytes by antibodies to galactocerebrosides. Kinetics of virus production showed that the release of virus peaked in the range of 10^5-10^7 PFU/ml or $MICLD_{50}$/ml. Neurons prepared from the spinal cord of rat embryos also exhibited a high susceptibility to CVS rabies virus infection without prior adaptation (139). The growth curve peaked at over 10^7 PFU/ml. Rat DRG neurons were also

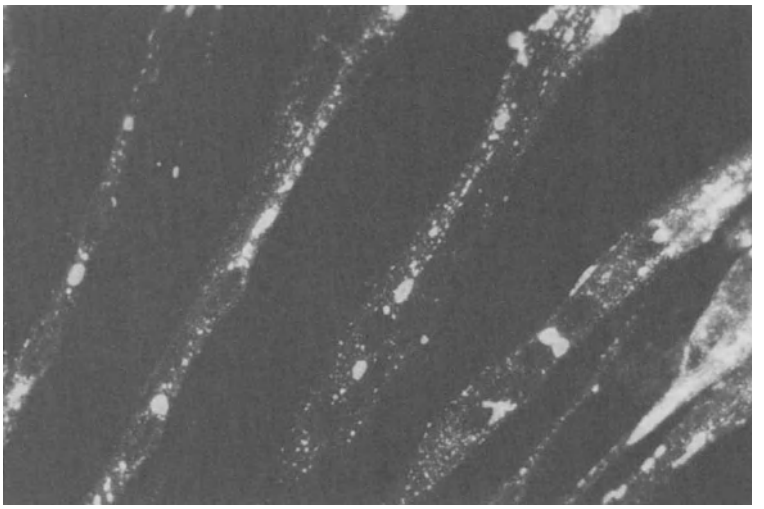

Figure 3. Infection of differentiated rat myotubes with street rabies virus isolated from fox salivary glands. Dissociated myotubes were obtained from the gastrocnemius muscle of 18 day-old rat embryos (139). Cultures infected with a street rabies virus from fox salivary gland (10^5 $MICLD_{50}$/ml) were stained with an antirabies nucleocapsid fluorescent conjugate 4 days post-infection (x 1,600).

susceptible to rabies virus infection (Fig. 2); however, the peak of maximum virus production was somewhat lower than the one found for central and spinal cord neurons (77).

Myotropism

After peripheral inoculation of mice with rabies virus, the presence of viral antigens was demonstrated by immunofluorescence to persist locally for only a few hrs (116). This fluorescence disappeared by 12 hours after inoculation. Infectious virus particles could be recovered immediately at the site of inoculation (footpad) with a street rabies virus isolate. However the presence of infectious virus was transient and no infectivity was recorded 24 hours after inoculation (10). In other experiments, the persistence of virus was shown to be somewhat longer (43,127).

The finding that non neural cells (mainly myocytes) were infected locally both by fixed and street rabies viruses (21,93,94,95) identified muscle as a site of early viral replication. These observations

led to the hypothesis that rabies virus replication in striated muscle cells represents a local amplification step. Indeed, muscle spindles and tendon spindles, which possess high concentrations of nerve endings and nerve fibers, may well be the pathway to the peripheral nervous system (117). The possible involvement of motor end plates in striated muscle has been suggested (44). Fluorescent studies were not conclusive but electron microscopy has permitted demonstration of infection of motor end-plates in the masseter muscle.

These observations raised the question of the relative susceptibility of muscle cells to rabies virus infection and whether an amplification step is needed for the subsequent infection of the peripheral nervous system. Cultured rat myotubes from 18 day-old embryos were infected either with the CVS strain of fixed rabies virus or with a street rabies virus isolate from fox salivary glands (139,141) (Fig. 3). Both viruses were capable of infecting cultured differentiated myotubes; however, the susceptibility to CVS was rather low (virus concentrations in the range of 10^7 to 10^8 PFU/ml were necessary) as compared to that of the fox rabies virus isolate (virus concentrations in the range of 10^5 to 10^6 $MICLD_{50}$). Production of infectious virus particles was very different in these 2 strains: whereas fixed rabies virus was not productive, the fox rabies virus isolate yielded virus titers of about 10^5 $MICLD_{50}$/ml. Thus, it seems that fixed rabies virus does not undergo an amplification step at the site of virus inoculation whereas street virus (fox rabies) may well be amplified locally before entering the peripheral nervous system. The mechanisms and significance of the abortive replication of fixed rabies virus in the rat myotube are not understood. Abortive infection may provide the possibility for rabies virus to persist locally for long periods of time at the site of virus entry, and thus could be an explanation for naturally occurring rabies cases with prolonged incubation periods.

The rat skeletal muscle cell line L8 and its derivative L8 C13U have also been shown to be infected by the CVS strain of rabies virus although the capacity of these to produce infectious virus particles has not been reported (113). Cultured embryonic chick myotubes are also susceptible to rabies virus infection (69). Inoculated rabies virus was distributed as patches on the cell membrane, similar to those corresp-

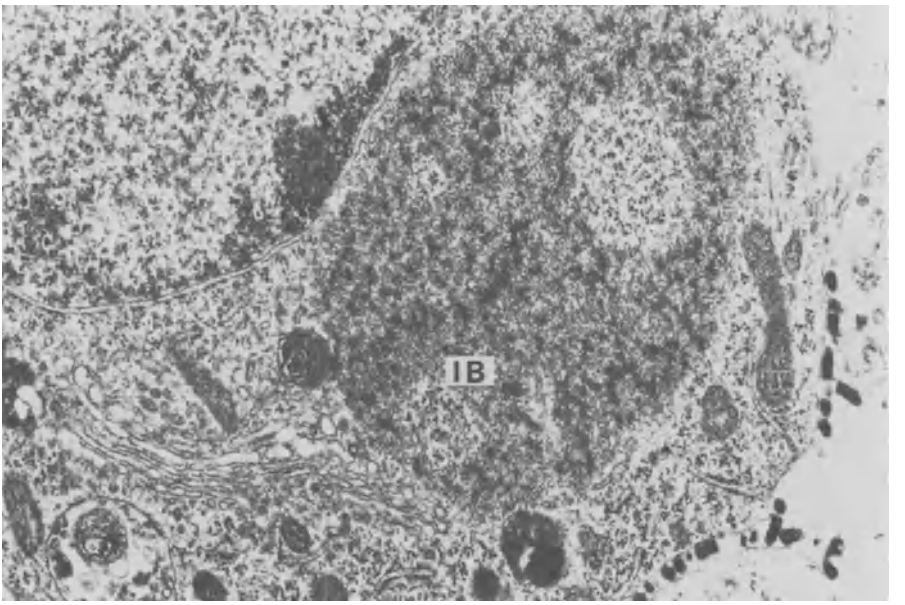

Figure 4. Infection of rat embryonic neurons from the cortex. Dissociated neuron-enriched cultures infected by CVS virus for 48 hrs. Inclusion bodies (IB) are heterogeneous and contain electron-dense structures. Virions are located in extracellular spaces and not in the intracellular compartment (x 20,000).

onding to rhodamine-labeled bungarotoxin (BTX), suggesting aggregation of virus particles at neuro-muscular junctions.

The infection of myotubes must be related to the putative role of the nicotinic acetylcholine receptor as a co-receptor for rabies virus. This is described later.

Morphology of Rabies Virus Infection in Cultured Cells

The earliest observations on the presence of rabies virions and inclusions in infected cultured cells were reported by Almeida *et al.* in 1962 (1), Atanasiu *et al.* in 1963 (7), and Matsumoto and Kawai (86). The morphogenesis of rabies virus has been investigated mainly in BHK-21 cell lines (8,51,57) and showed budding of virions from cell membr anes. Other studies have utilized cell lines derived from fibroblasts (28,53,129) and from murine neuroblastoma (53), as well as from organized cultures of mammalian neuronal tissues (87,88) and primary

neurons from dissociated neural tissues (superior cervical ganglia (140), spinal cord (139)). In these *in vitro* systems, the formation of virions occurs in association with either the plasma membrane or the various membranes of the intracellular compartment. Formation of virions in either site depends upon the cell type and the viral strain as well as the degree of infection. The combination of these parameters gives a variety of modes of viral production, as demonstrated in the above studies.

From the various cell systems we used, those which produce high yields of infectious viruses usually contain large heterogeneous inclusions containing dense structures. This is the case not only for infected dissociated neurons from the cortex (Fig. 4), and the spinal cord (139), but also from rat DRG (Fig. 6) which is less productive. In contrast, dissociated cells which do not produce significant yields of

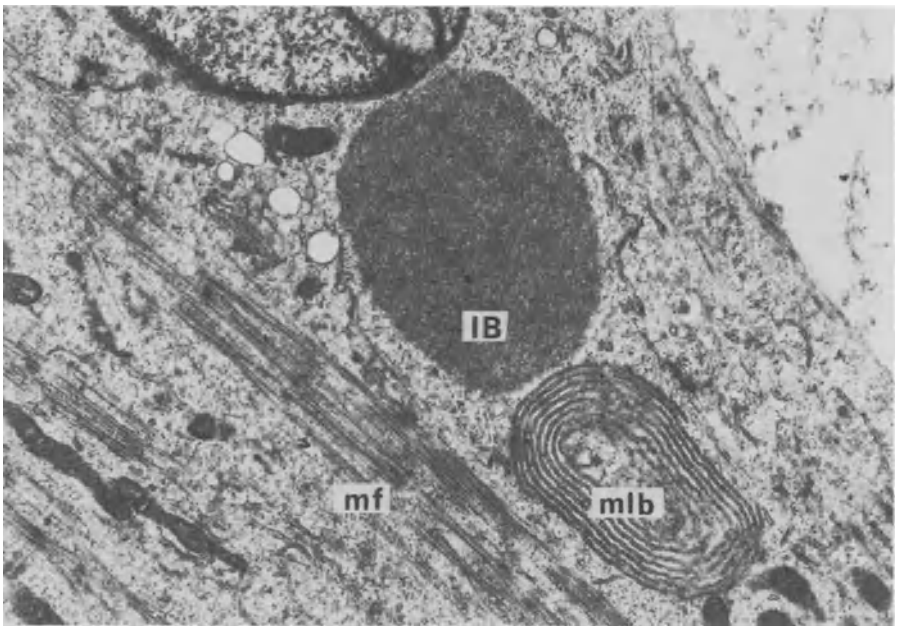

Figure 5. Infection of rat embryonic differentiated myotubes. Differentiated primary cultures of rat myotubes were infected for 72 hrs. Myofilaments (mf) are present in the cytoplasm. Homogeneous inclusion bodies (IB) characteristic of rabies virus infection are observed in the absence of virions, either in the cytoplasm or extracellularly. A multilamellated body (mlb) is also seen in the cytoplasm (x 10,000).

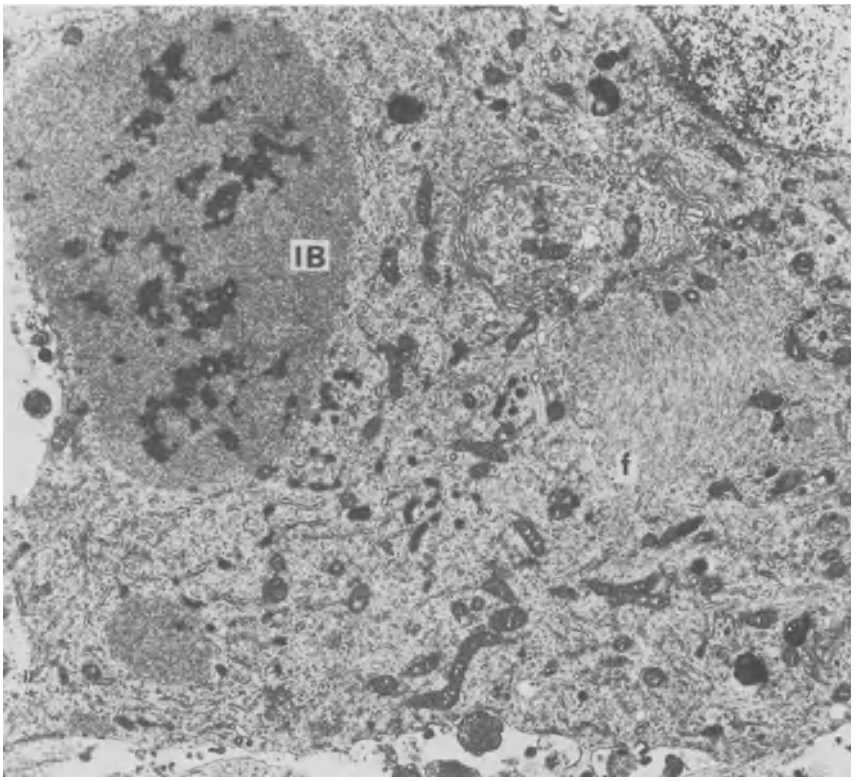

Figure 6. Infection of rat sensory dorsal root ganglia. Rat DRG neurons were infected with CVS virus for 6 days. Inclusion bodies are heterogeneous and virions are present in the endoplasmic reticulum cysterna. Bundles of fibrils (f) are observed in the cytoplasm (x 11,200).

infectious virus usually show the presence of homogeneous inclusions. This is the case for cultured myotubes which support an abortive infection by CVS fixed rabies virus (Fig. 5) or neurons from the superior cervical ganglia (140). Multilamellate bodies have also been found in infected myotubes (Fig. 5). These structures have also been described by other authors (133).

As for the formation of virus particles, neurons from the cortex, which are the most efficient cellular systems for the production of infectious virus without prior adaptation, appear to contain no intracellular virions, whereas a large number of virus particles can be observed in extracellular spaces (Fig. 4). In DRG neurons, which are

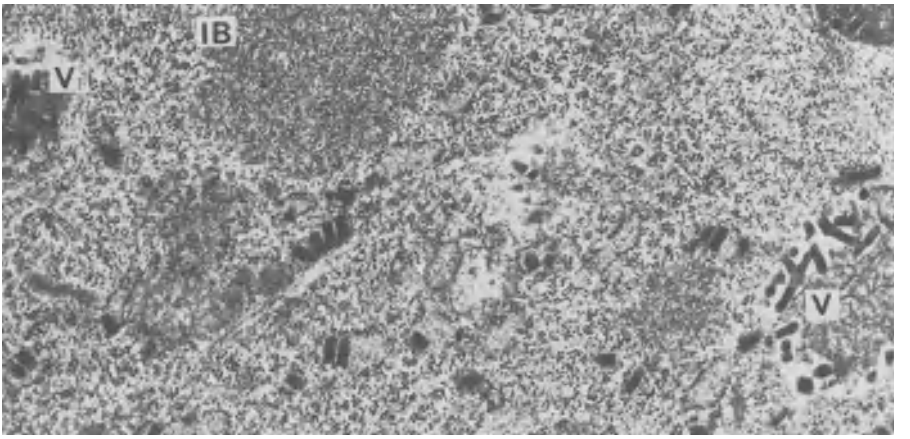

Figure 7. Infection of CER fibroblasts. The chicken embryo-related (CER) cell line is naturally susceptible to rabies virus. 48 hrs after infection with CVS virus, virions can be observed either in vacuoles (V), close to the inclusion bodies (IB), or in the endoplasmic reticulum (x 18,400).

relatively less productive than cortical neurons (77), virions can be observed at the level of the endoplasmic reticulum (Fig. 6). Infected fibroblast cell lines such as CER present a variety of intracellular viral production sites (Fig. 7).

From these observations, it appears that the presence of dense structures in the inclusions is related to the production of high yields of infectious viral particles. This is the case for the cortical neurons in which viral exocytosis occurs rapidly without accumulation of virions in the cytoplasm. It results in high yields of virus in the supernatant. In contrast, neurons which produce lower yields of virus, such as those from DRG, do not contain dense structures but have a tendency to accumulate the virions in the reticulum.

ATTACHMENT OF RABIES VIRUS TO HOST CELLS

The very first event in viral infection is the contact of the virion with cellular membrane structures. This allows binding and subsequent internalization of virus into the intracellular compartment. Attempts have been made to characterize a cellular receptor to rabies

virus by approaches including: (i) binding of rabies virus to cells or to cellular components; (ii) inhibition of viral binding after enzymatic treatment of cell surface; and (iii) competitive inhibition of viral binding by molecules that compete for cell membrane receptors. Most investigations fall into one or another of these 3 categories regardless of the cellular substrate used. BHK-21 cells have been widely used, as well as membranes derived either from BHK-21 cells or from the CNS. There is a recent trend to use natural target cells for rabies virus (cultured neurons and muscle cells). These cells are more tedious to cultivate but reflect more accurately the properties of the natural host cells during the viral ascent from the periphery to the CNS.

Binding Studies

Metabolically-labeled rabies virus has been shown to attach to the following cell lines: BHK-21, mouse neuroblastoma NA, and rat skeletal muscle (70,110,111,154). Saturation of virus binding on the cell surface was obtained with 3-5 x 10^3 attached virions per cell (154). Competition for rabies virus binding sites occurred between the rabies apathogenic variant virus RV194-2 and vesicular stomatitis virus (VSV) but failed to occur with other neurotropic viruses (e.g., reovirus type 3). Rabies virus glycoprotein also failed to compete under similar conditions (154). ^{125}I-labeled rabies virus also binds to another clone of murine neuroblastoma cell line (NS20) (137). Preincubation of NS20 cells with an excess of unlabeled rabies virus resulted in a dose-dependent inhibition of label binding. Binding of rabies virus to several cell membrane preparations from various cell types has been investigated (70). Myotubes, neuroblastoma and salivary gland preparations had the greatest binding activity for rabies whereas striated muscle and nerve membranes had an intermediate effect. The lowest binding activity was found with membranes from parenchymal tissues, erythrocytes and other tissues. Purified synaptic membranes competed with rabies virus resulting in a 80% inhibition of infection of target cells (Fig. 8). Radioiodine-labelled rabies virus also bound to isolated synaptic membranes from rat spinal cord, immobilized on CNBr-activated beads of Sephadex G-200 (Tsiang & Bizzini, unpublished).

Binding studies are difficult to interpret, however, since they do not discriminate between infectious virions and inactivated ones. In

fact, infectious virions also bind to non-susceptible cells (L cells, MDBK cells) or bind poorly to susceptible cells (neuroblastoma cells, primary neurons) which have very efficient internalization and replication mechanisms (Tsiang, unpublished).

Enzymatic Treatment of Target Cells

Different enzymes have been used to treat susceptible target cells before infection with rabies virus. The use of glycosidases has shown that neuraminidases have an inhibitory effect. Neuraminidase at concentrations in the range of 10-20 U/ml repeatedly reduced the susceptibility of various cell types, including fibroblastic lines (128,129) and a variety of cells of different origins: neuroblastoma cell lines, and primary neurons and myotubes (Goldoni & Tsiang, unpublished). However, the neuraminidases were not all identically active: neuraminidase from *Cl. perfringens* was active whereas the enzyme from *V. cholerae* was not (128). Recovery of cellular susceptibility occurs after a 5-6 hr incubation in the absence of enzyme (129). In CER cells, beta-galactosidase also had an inhibitory effect and alpha-mannosidase had an intermediary activity, while fucosidase had no effect in preventing rabies virus infection (24). Studies of the role of cellular lipidic structures on the binding of rabies virus showed that phospholipase treatment of CER cells decreased the viral attachment; however, only phospholipase A2 had a dose-dependent effect (130). At a 10 U/ml concentration, this enzyme inhibited 80% of infection as monitored by fluorescence. The recovery of susceptibility to infection was total when CER cells were incubated for 6 hrs in the absence of phospholipase A2. Sphingomyelinase did not reduce CER cell susceptibility to rabies virus infection, in contrast to its inhibitory action on VSV infection of these cells (130). In all enzyme treatment experiments, no inhibitory effect was ever found when glycosidases or phospholipases were incubated with the cells immediately after the removal of the viral inoculum.

Trypsin, proteinase K and pronase have been reported to have no effect on viral attachment to cells (129), and cellular susceptibility to viral infection was even enhanced after subtilisin treatment. Thus, external membrane proteins do not appear to participate in viral attachment, but later involvement of cellular proteins cannot be ruled

out, since the subsequent virus internalization mechanism may need the participation of cellular proteins which may not be accessible to proteases.

Molecules that Compete with the Binding of Rabies Virus to Cells

Competition between virus binding to cells and presumably active molecules has been employed to determine the cellular surface components that serve as receptors for rabies virus. Such molecules have been used either in purified form from commercial sources, or have been extracted from cellular substrates. Special attention has been given to cellular gangliosides and to the acetylcholine receptor which has been shown to have an active role in the early steps of rabies virus replication.

Competitive binding of various phospholipids was tested in CER cells. Mixtures of phospholipids and rabies virus were prepared and applied immediately, or after 2 hrs' incubation, to CER cells. Of the different phospholipids, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS) and sphingomyelin (SM), only PS was active. A 90% inhibition was obtained with 500 μM PS (130). It is interesting that identical results have been observed with VSV (115,130).

Concanavalin A (con A) has inhibitory effects on *in vitro* infections by viruses, mainly by binding to cellular membranes (102). More specifically, it binds to alpha-D-mannopyranosyl and alpha-D-glucopyranosyl residues of carbohydrate-containing components on the cell surface (42,121). Pretreatment of CER cells with con A reduced the cellular susceptibility to rabies virus infection (25). The lectin was also found to be inhibitory when applied after the viral binding step. These inhibitory effects were reversed by alpha-D-methyl-mannopyranoside. These data suggest that carbohydrate moieties in addition to sialic acid may play a role in the early steps of rabies virus infection. Other lectins, including wheat germ hemagglutinin (WGA) which reacts with N-acetylglucosamine, limulin which reacts with N-acetylneuraminic acid, and the lectin from *Lotus tetragonolobus*, were also tested. While WGA and limulin exhibited an inhibitory effect, the lectin from Lotus which specifically reacts with fucose residues had no effect (25).

Competition experiments were also performed with synaptic membranes prepared from rat brain homogenates. Incubation of rabies virus with synaptic membrane prior to infection of target cells resulted in a considerable reduction in the percentage of infected cells as monitored by fluorescence 24 hrs after viral inoculation (Fig. 8).

Data are accumulating to support the participation of cellular phospholipids and/or glycolipids in the binding site of rabies virus (110,154). A chloroform/methanol-soluble extract obtained from octylglucoside-solubilized BHK-21 membranes has been shown to block rabies virus binding (154). The inhibitory effect of this extract resisted protease treatment but was destroyed by phospholipases and neuraminidase. Extraction of an inhibitory cellular component was also obtained

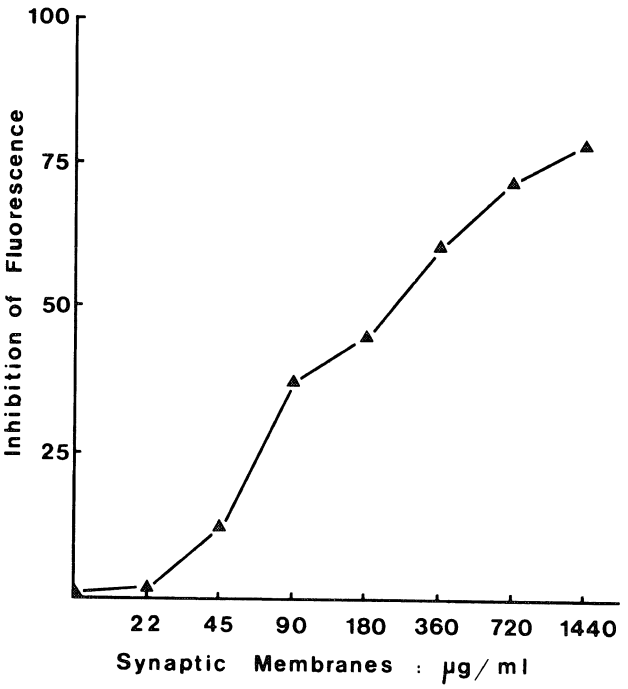

Figure 8. Inhibition of rabies virus binding to susceptible cells by rat synaptic membranes. For the preparation of synaptic membranes, rat brains were homogenized in a buffer containing protease inhibitors; the extract was centrifuged in a Ficoll-sucrose gradient and processed as described by Monneron (90). Rabies virus (10^6 PFU/ml) was incubated at 37°C for 1 hr with different concentrations of synaptic membrane prior to infection of CER cells (1 hr at 37°C) as described previously (129). The effect is measured as percent inhibition of the number of fluorescent (infected) cells with respect to controls (Ermine, d'Aloyer and Tsiang, unpublished).

from a rat membrane preparation solubilized with octylglucoside. Further purifications showed that this extract contained mainly lipidic structures (24).

The Nicotinic Acetylcholine Receptor and Rabies

The hypothesis of a nicotinic acetylcholine receptor (AChR) as a co-receptor for rabies virus arose from experiments showing: (i) accumulation of rabies virus at the neuromuscular junction on dissected mouse diaphragm suggesting the AChR to be a site of preferential binding for rabies virus (69); (ii) competitive binding of cholinergic antagonists (alpha-bungarotoxin (BTX) and D-tubocurarine) and rabies virus to cultured chick embryo myotubes (69); (iii) competitive binding of BTX and rabies virus to cultured rat myotubes (139); (iv) characteristics of the attachment of virus to membranes prepared from chick embryo myotubes (70); (v) attachment of rabies virus to affinity-purified AChR from Torpedo electric organ in a solid-phase binding assay (68); and (vi) the observation that the highly conserved residues of the curaremimetic neurotoxins from snake venom, which bind to the AChR site, have amino acid sequence homologies with a segment of the rabies virus glycoprotein (70,71). These data argue that the AChR region may be involved in the binding for rabies virus, especially in muscle cells which contain large amounts of receptors and presumably represent the primary target cells in naturally occurring rabies.

On the other hand, it must be noted that BTX is efficient only at high concentrations (10^{-5} to 10^{-7} M), and its inhibitory effect is usually incomplete (69,139). Other cell types lacking nicotinic AChR are also susceptible to rabies virus infection, demonstrating that the AChR is not an obligatory necessity (112,139). For instance, rabies virus infection of neurons which are highly susceptible is not inhibited by antagonists to either nicotinic AChR (BTX) or muscarinic AChR (scopolamine) (Tsiang, unpublished data). Other cellular structures may also be involved in the viral attachment. As noted elsewhere in this chapter, carbohydrate moieties, phospholipids and highly sialylated gangliosides may also participate as components of a complex receptor for rabies virus.

The Role of Gangliosides in Rabies Infection.

Among the lipidic structures involved in the early steps of rabies virus infection, gangliosides are potential candidates. These glycosphingolipids are distributed ubiquitously on the membranes of many cell types. They are characterized by the presence of 1 or several sialic acid residues. Gangliosides, however, are most abundant in cells derived from brain structures (3,67), and they are assumed to be important structures in cell surface events (155). Enveloped RNA viruses have been reported to bind to gangliosides on the surface of susceptible cells (43,84,131). Therefore the contribution of cellular gangliosides to rabies virus infection was investigated.

The insertion of sialic acid or gangliosides into the cell membrane of desialylated cells restored their initial susceptibility to attachment by several viruses (47,84,108). In our experiments, neuraminidase-treated CER cells, which had lost their susceptibility to rabies virus infection, were incubated with gangliosides. Incorporation

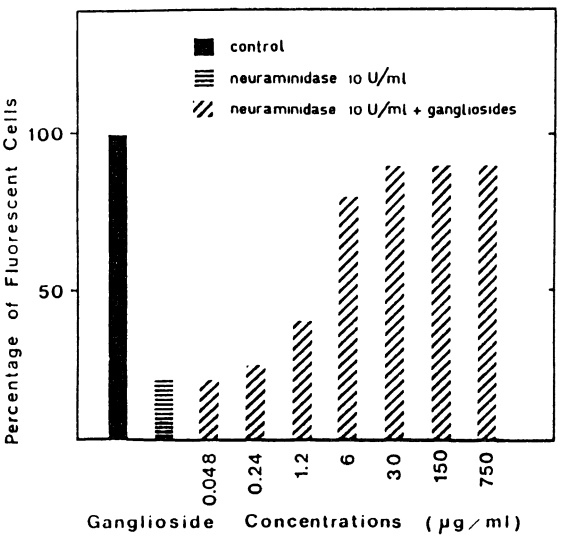

Figure 9. Recovery of susceptibility to rabies virus infection by incorporation of gangliosides into desialylated cells. CER cells treated with *Cl. perfringens* neuraminidase (10 U/ml) (horizontal bars) showed a decreased susceptibility to rabies virus infection with respect to the virus control (black bar). Incorporation of increasing concentrations of gangliosides into the desialylated cells resulted in recovery of susceptibility to rabies virus infection.

of gangliosides into the cell membrane of desialylated CER cells allowed them to recover their initial susceptibility to the viral infection (Fig. 9). The use of single purified gangliosides or mixtures of 2 gangliosides to restore cellular susceptibility to viral infection showed that GT1b and GQ1b, which contain several neuraminic residues, were the most effective. The disialogangliosides, principally GD1b, were also active whereas GM1 and GM3 were poorly active and GD3 inactive at the same concentrations (128). From these data, the activity of gangliosides appeared to be related to the length and conformation of the carbohydrate chain. The presence of a sialylated group on the external and internal galactose was necessary, whereas the presence of 2 sialylated residues on the internal galactose appeared to be the most efficient structure. This is the case for GT1b and GQ1b. However, the recovery of cellular susceptibility was never total with individual gangliosides, suggesting that other cellular components, or other phenomena, are also involved.

In another series of experiments, cellular components prepared from normal rat CNS membranes were shown to be active in competitive binding of rabies virus to fibroblasts and neurons (24). Detergent extracts from these membranes were analysed as to their capacity to compete with rabies virus binding. The gangliosides extracted from these CNS membranes were active in both target cells. These data provide additional arguments for the involvement of gangliosides in rabies virus infection of target cells. It is interesting to note that high levels of gangliosides are found in nervous tissues (3,67,132), particularly GT1b, and GQ1b which are present in higher concentrations in neurons than in glial cells (78). The observation that virions are often seen near synapses (18,21), which are structures containing important quantitities of highly sialylated gangliosides (72), may also be pertinent.

In conclusion, the receptor to rabies virus seems to be a complex structure in which any eventual role of cell membrane protein has not been demonstrated. Cell lipidic structures seem to be involved in rabies virus infection. Carbohydrate moieties play a determining role, mainly in highly sialylated structures such as gangliosides. In our opinion, the specificity of gangliosides as receptors for rabies virus

could be limited to their role as vehicles for sialic acid residues to be presented in an adequate conformation. On the other hand, the receptor to nicotinic acetylcholine may well play a role in specific cells (muscle), but may not be the only molecule involved in a complex receptor for all other susceptible cells (neurons). Therefore, it is predictable that the receptor for rabies virus may vary from one cell type to another and to consist of local and transient conformational micro-environment with regard to the fluidity of the host cell membrane. Isolation of such a non-specific receptor complex, which might furthermore interact with active molecules, would be a difficult task.

ENTRY OF RABIES VIRUS INTO THE HOST CELL

Virus penetration into the host cell occurs either by adsorptive endocytosis or by direct fusion with the cellular membrane. In infection of BHK cells by rabies virus, both fusion and viropexis have been described (89,110). Cell fusion activity by rabies virus is also associated with a pH-dependent hemolysis, both phenomena being related to the mechanism of viral entry (79,89).

Kinetic studies using electron microscopic examination have shown the attachment of rabies virions to microvilli of CER cells. Virions were frequently associated with coated pits. Five minutes post-infection, coated or uncoated vesicles contained 2-5 virions. The number of virions per vesicle increased, and during the following minutes, they were detected in large vacuoles that were identified as prelysosomes. Finally, virions were present as aggregates in large lysosome-like vacuoles. Occasionally, the viral membrane was observed to fuse with the vacuole membrane (129). Virions associated with coated pits were also seen in cultured neurons (140).

To test the role of lysosomal fusion in the entry of rabies virus into CER cells, ammonium chloride and chloroquine were used to raise the intra-lysosomal pH in order to prevent fusion from occurring (129). Adding these agents in the culture medium immediately after the viral attachment step totally inhibited viral replication. When these agents were added to the medium at different time intervals post-infection, kinetic studies showed that the inhibitory effect occurred early during the uptake of the virus. Addition of ammonium chloride

6 hrs post-infection had no effect. This suggests that the drug was only active during the first few minutes after viral entry and supports the argument of a lysosome-dependent fusion step during rabies infection. Ammonium chloride and chloroquine were also active in other cell types: murine neuroblastoma cell lines (NS20) (143), cultured primary myotubes, and different neuronal cell types from embryonic rodents (Tsiang, unpublished). These findings suggest that the lysosomal pathway is a general route of entry of rabies virus into the host cells.

TRANSPORT OF RABIES VIRUS IN THE NERVOUS SYSTEM

Migration of rabies virus in the peripheral nerves was postulated by Morgagni in 1769 (91). Rabies virus was successfully isolated from peripheral nerves in experimental rabies by Roux (114). Transmission of rabies by direct inoculation of the viral inoculum into nerves was achieved by DiVestea and Zagari in 1889 (31). These authors recovered the virus from the spinal cord. They also found that amputation of an infected leg interrupted the progression of the disease, thus confirming the initial hypothesis of Morgagni. Interruption of peripheral nerves corresponding to the inoculation site protected against rabies virus challenge, as demonstrated by many authors (46,99,100).

A systematic study of the distribution of rabies virus suggested the transport of rabies virus by nerve fibers after intramuscular inoculation of virus into the masseter muscle. Virus was recovered from the sensory part of the trigeminal nerve and in the ganglion of Gasser (39). More recent studies also confirmed these early experiments by sectioning the nerves or by amputation of the limb (9,10,26,58) and by ligature of the nerve (8). However, sectioning and ligatures interrupt not only the axoplasmic flow but also the integrity of other cell types present in the nerve (Schwann cells, supportive cells). Thus, the use of compounds such as colchicine and vinblastine which act on microtubules and which specifically interrupt axoplasmic flow has implicated more definitively the intra-axonal route of rabies virus migration to and from the CNS (16,135). The subsequent transport of rabies virus from the intervertebral spinal ganglia to the CNS seems to occur through the dorsal roots (118).

On the other hand, it is still controversial as to whether sensory or motor fibers are involved in the viral transport. Dean *et al.* (26) reported that sectioning of either sensory or motor roots failed to prevent the viral spread, while section of the peripheral nerve containing both fibers prevented its spread. This finding favors the hypothesis that both sensory and motor fibers are potential routes of entry for rabies virus.

Transport of Rabies Virus in Neurons

Further details on the axoplasmic transport of rabies virus have been reported using differentiated rat sensory dorsal root ganglia (77). Use of a 2-chamber culture device, originally designed by Campenot (20), allowed us to infect separately either the neuronal soma and neurites extending from these cells (in a cylinder), or only the neurites which crossed a silicone grease barrier (outside the cylinder). Infection of the neuritic compartment and the subsequent

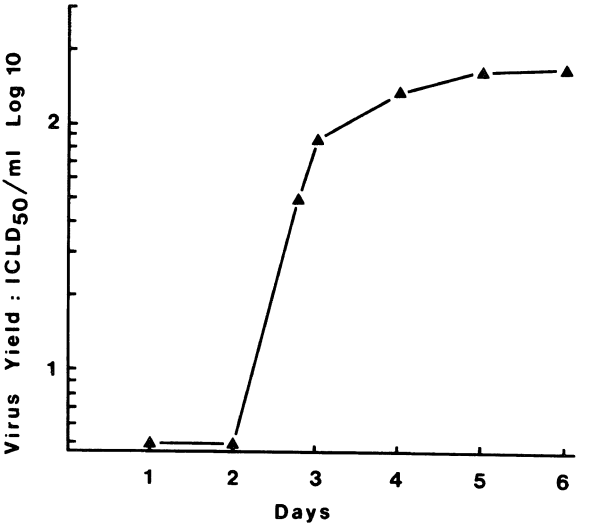

Figure 10. Retrograde transport of street rabies virus in DRG neurons. DRG neurons from 14 day-old rat embryos were seeded in a cloning cylinder and the neurites allowed to extend across the cylinder into the outer compartment as described elsewhere (77). After inoculation of virus (10^7 $MICLD_{50}$/ml) in the outer compartment, the neurites were destroyed with sulfuric acid. The supernatant in the cloning cylinder was sampled every 24 hrs, and titrated in mice for infectious virus.

recovery of the viral infectivity in infected DRG cells in the cylinder showed that the rate of axoplasmic transport is in the range of 12-24 mm/ per day (*cf.* 75 mm/ per day observed *in vivo* by Dean *et al.* (26)). These experiments demonstrated the capacity of cultured sensory neurons to transport rabies virus (CVS) by retrograde axoplasmic flow. Infection still occurred even in the presence of anti-rabies serum in the inner cylinder, showing that rabies virus is capable of infecting a neuronal network under the modulatory effect of the B cell-dependent immune response (77). Other authors have reported conflicting results on the capacity of immune antibodies to inhibit viral spread from cell to cell (30,75). However, even if circulation of virus in the neuronal structure is not affected, this does not necessarily rule out the capacity of the immune response to modulate the expression of viral antigens on the cell surface as demonstrated for other enveloped viruses (33,103). Both colchicine and cytochalasin B inhibited virus transport, thereby demonstrating the participation of microtubules and structures containing actin. Street rabies virus (a salivary gland isolate from infected foxes) is also transported by retrograde axoplasmic flow in the cultured rat sensory neurons (Fig. 10).

The anterograde transport of rabies virus can also be demonstrated in this compartmentalized culture system; however, its mechanism is probably complex, involving a direct and immediate uptake and delivery of virions from 1 side of the barrier to the other side without any replication of rabies virus. A second mechanism most probably involves the transport of rabies virus which has replicated in the cell soma before delivery at the nerve endings (data not shown).

Transport of Rabies Virus in the Brain

As suggested by electron microscopic studies, viral spread in the CNS occurs either by travel in extracellular spaces after budding of the virions at the surface of infected cells, or via neurites (52,55, 56,96). Neural spread is suggested by observations that the sequences of infection of brain nuclei differ according to the route of virus inoculation (32,66,134). The access of target organs to the viral infection is also dependent on the viral strain (29,66). The stereotaxic inoculation of rabies virus into the rat neostriatum followed by the detection of rabies inclusions only in physiologically connected

areas (substansia nigra, cortex, thalamus), after a single virus growth step is an argument supporting retrograde axoplasmic transport of rabies virus as the first mechanism to occur (37).

INTERFERON IN RABIES VIRUS INFECTION

The involvement of an interferon-like substance in rabies was suspected by Louis Pasteur three-quarters of a century before interferon (IFN) was actually discovered. Pasteur observed that repeated shots of rabies vaccine prepared from infected spinal cord would enhance the protective effect and that some unidentified compound in the nervous tissue accompanying the attenuated virus conferred a non-specific protection (105).

The production of IFN has been demonstrated in experimental rabies infection (61,135,152). Its action has been shown to be related to the activation of two IFN-mediated enzymes, 2-5A synthetase and protein kinase (p67K) (83). Two peaks of IFN levels were detectable in the plasma, the first one corresponding to the presence of rabies virus at the peripheral site of virus inoculation, and the second to the presence of virus in the brain. The early production of IFN could be neutralized by anti-mouse alpha-/beta-IFN globulin which modified the development of the disease by shortening the morbidity period (80).

Induction of high titers of IFN in the brain, however, does not result in an inhibition of viral replication in mice (81). Induction of IFN and IFN-mediated enzymes in the CNS has been shown to vary in different dissected brain regions. The quantitative presence of rabies virus in different brain areas did not correlate with the levels of IFN, showing that the capacity of different neuronal populations to respond to rabies infection varies from one neuronal area to another (82).

Interferon production has been shown to occur in infected cultured cells (152,157). On the other hand, treatment of tissue cultures with exogenous IFN protects them from rabies virus infection. This protective effect has been observed in cells of various origins: canine (27), hamster (151), mouse (12), and in human diploid cells (152). Established murine and human neuronal cell lines are also protected by exogenous IFN from rabies virus infection (Fig. 11).

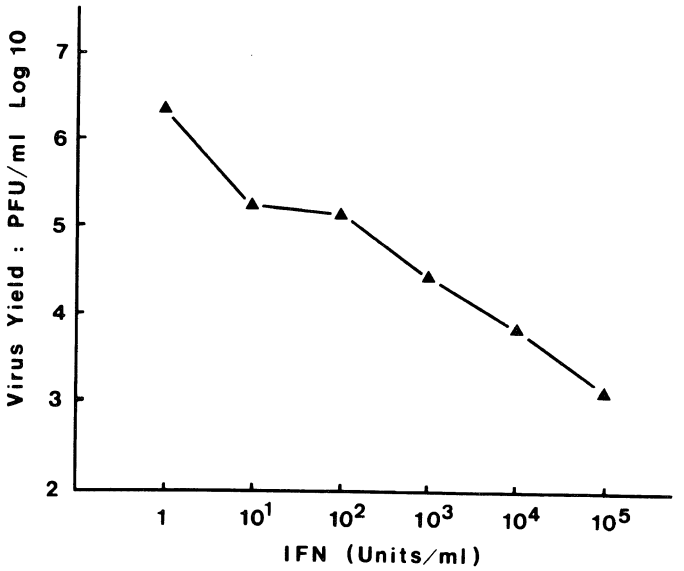

Figure 11. Effect of human lymphoblastoid IFN on rabies virus infection of IMR 32 human neuroblastoma cells. Monolayers of neuroblastoma cells were treated with different IFN concentrations for 18 hrs at 37°C. The cells were washed twice and infected with a m.o.i. of 0.5-2 PFU/cell. After 30 hrs, virus yields from the supernatants were titrated by plaque assay.

Neurons have the potential capacity to respond to the antiviral effect of IFN. Treatment of mouse neuroblastoma cells with murine IFN-alpha/beta resulted in a dose-dependent response to the 2-5A synthetase and protein kinase p67 (50). Rabies virus-infected neuroblastoma cells also show a decrease in the viral yields after IFN treatment. These data do not explain the observed ineffectiveness of IFN treatment against rabies viral infection in immunocompetent mice. However, IFN certainly has an efficient effect in mice with an impaired T cell-mediated immune response (50).

Interferon may also be involved in persistent rabies infections. In BHK-21 cells persistently infected by rabies virus, Kawai *et al.* (62) failed to detect any interferon (IFN). But in a persistently infected human neuroblastoma cell line (K-104) which originated from the SYM-I strain, rabies virus persistency was demonstrated to be

mediated by endogenous interferon production (48). The authors suggested that the virus-induced interferon in K-104 cells affected rabies virus replication in these cells and subsequently reduced the progeny virus yield. These studies were further confirmed by the observation that anti-interferon antibody increased the viral replication and markedly enhanced the cytopathic effects (49). During these experiments, small plaque mutants appeared which replaced the original virus during long term cultivation of the persistent infection. The authors postulate that such mutations generate population variations in prolonged and/or latent infections, and that such persistency could be a mechanism for the development of latent infections (49).

FUNCTIONAL ALTERATIONS IN RABIES VIRUS INFECTION

Infection of cultured cells by rabies virus can result in extensive cytopathic effects (6,34,35) or none at all (19,36), although cellular metabolism may be altered despite the absence of morphological changes. A report indicates activation of lysosomal enzymes in rabies virus-infected cells (29). Acutely infected primary neurons can harbor the virus for over 1 month without showing any morphological modifications (77). Thus, cell death does not seem to be a prerequisite for rabies pathogenesis. On the other hand, the specific rabies histopathological lesion, the Negri body (98), is an inconstant one. From various authors, Negri bodies are present in 66-93.3% of cases of clinical rabies (4). Non-specific histological findings are more frequent although these may vary with species and viral strain. It was originally controversal as to whether the Negri body contained rabies virus (153) or whether it was a degenerative structure of infected cells (124). It is now generally accepted that the Negri body contains viral material (38,51,58,85,97). Usually, more severe inflammatory reactions are found in field rabies virus infections (93). The immunopathological events seem to indicate that an adverse immune response modulates the clinical aspects of the disease (e.g., disappearance of paralysis (41,54,122) and the "early death" phenomenon (17)), but does not modify the lethal outcome. These observations support the contention that virus-induced alterations of specific brain functions occur during the disease. Among these neuronal functions several can be

investigated with regard to viral infections: (i) neurotransmitter metabolism (synthesis, transport, release, degradation); (ii) neurotransmitter receptor functions; (iii) differentiation with respect to cellular parameters; (iv) specific neural protein biological activity; and (v) electrophysiological parameters.

The possible involvement of monoamine metabolism in rabies infection has been investigated in a comparative study with herpes infection. Some modifications were detected in the release and synthesis of dopamine and 5-hydroxytryptamine (5-HT), while of the acid metabolites homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA), the latter was increased (76). Another report indicates the depletion of noradrenaline (NA) and dopamine whereas the 5-HT concentration was unchanged (11). Further detailed studies are needed to determine the virus induced changes in neurotransmitter metabolism in defined brain regions.

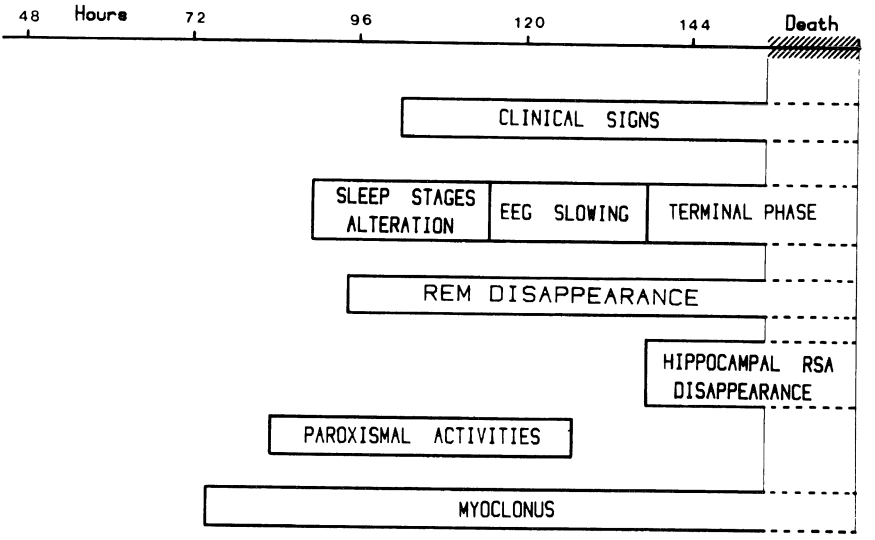

Figure 12. Sequential development of EEG sleep changes and clinical changes in CVS rabies virus-infected mice. The kinetics of appearance of the disturbances were remarkably constant for different mouse strains (C57B1/6, DBA/2, C3H/He, Balb/c, CBA). The REM sleep disappeared in the early stages of EEG alterations, whereas the hippocampal RSA was still preserved until the terminal phase. Paroxysmal activities also appeared during the course of the disease.

In vitro studies have mainly focused on alterations in neurotransmitter receptors. A mouse neuroblastoma-rat glioma hybrid cell (108-CC-15) has shown a lower stimulation of cAMP synthesis by PGE1 either in acute or persistent infection by the HEP-Flury strain (65). In both cases, an increase in the dissociation constant for the agonist ^{3}H-etorphine was observed by Scatchart plot analysis; however, the number of opiate receptors per cell was not modified (92). In the same persistently infected NG-CC-15 hybrid cell line, the adenylate cyclase system has been reported to be involved in that there is inhibition of the stimulation of GTPase activity of the Ni (inhibitory regulatory component) (64). Also in the same cell line, the binding of an antagonist (^{3}H-scopolamine) to muscarinic AChR was also found to be impaired (138), thus confirming earlier *in vivo* studies (136). These data support the hypothesis that, in rabies viral infections, specific neural functions are modified during the disease.

Electrophysiological studies have shown the early occurrence of brain function alteration in chronically implanted mice infected with fixed rabies virus (40). The earliest phase which sometimes occurred before the onset of clinical signs showed alterations of sleep stages, the disappearance of paradoxical sleep, and pseudoperiodic facial myoclonus (Fig. 12). The mature phase was characterized by an EEG slowing (2-4 c/s) with a cortical activity which flattened at the terminal stage. The brain electrical activity ceased about 30 minutes before cardiac arrest, suggesting that cardiac failure may not be the direct origin of rabies death. The occurrence of the alterations in EEG and sleep stage organization were also quantified by spectral analysis. Identical data were found for experimental rabies in the cat (data not shown). Street rabies-mediated brain function alterations showed different patterns of evolution in mice and cats, suggesting different pathogenetic mechanisms for fixed and street rabies virus strains (data not shown). The finding of altered electrical activity in the CNS of experimentally infected rodents and carnivores constitutes an additional argument that rabies may be a disease of impaired information transmission.

CONCLUSIONS

Interaction of rabies virus with host cells is certainly one of the main fields of investigation for this viral disease. Because of the selective affinity of rabies virus for neuronal structures and particular features of the disease process, rabies is a unique example of neurotropic viral disease. There are many parameters associated with the tropism of rabies virus for neuronal structures: (i) the cellular susceptibility; (ii) the capacity of neuronal structures to support rabies virus replication; (iii) the viral transport in neurons; and (iv) the impairment of neural functions. These features are all important for the virus to modify the infected host so that the disease can be transmitted to an uninfected one: first by changing its behavior to a state of aggressiveness; and secondly by excreting the virus in the salivary glands, thereby permitting inoculation of the virus by bites.

Understanding the mechanisms of virus targeting and those of brain function alterations may provide new therapeutic tools against this disease by: (i) interrupting the virus spread with non-virucidal drugs acting on virus transport; and (ii) modifying the altered functions with specific molecules acting on neuronal functions. Thus, in addition to the classical treatment of rabies by active or passive immunization, the more specific aspects of mechanisms of the disease may be amenable to intervention.

The use of neural cell lines and cultured dissociated cells from tissues which constitute natural target organs for rabies virus introduce new prospects for research into the pathogenesis of rabies. It allows investigation of pathogenetic events which cannot be observed in other cell types such as fibroblasts. Moreover, cultured neurons are a basic cellular substrate for investigations with street rabies virus strains which do not replicate efficiently in cells of other origin.

Infection of primary neurons derived from different neural tissues (cortex, spinal cord, DRG, superior cervical ganglia) has shown that they exhibit different susceptibilities to rabies virus. Moreover, their capacity to replicate infectious virus particles also varies (e.g., DRG neurons have a moderate susceptibility and capacity to replicate rabies virus as compared with cortical neurons). On the other hand we have reported that cultured neurons may lose some of the

natural barriers that exist *in vivo* (e.g., neurons from the superior cervical ganglia are not infected by rabies virus *in vivo* but are susceptible to infection in cultures). Therefore, although cultured neurons are the best tools available, it is also necessary to be aware that brain nuclei represent highly organized and specialized neuronal structures which cannot be duplicated *in vitro*. In fact, correlation of data from both *in vitro* and *in vivo* approaches is required.

The impairment of altered functions in infected neurons may also be modulated by the T and B cell-mediated immunity or other non-specific immune defences (see Macfarlan, this volume). These complex interactions also need further investigations in cultured primary neurons.

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THE PATHOGENESIS OF RABIES

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INTRODUCTION

Most of our knowledge of the pathogenesis of rabies comes from research done since the late 19th century. As could be expected, advances have paralleled fairly closely developments in appropriate technology. Early studies established the neurotropism of the infectious agent and outlined the main routes of movement of virus through the animal body. Experimental infections of animals done in conjunction with or followed by various surgical procedures and virus isolation indicated the neural route to the central nervous system (CNS), the rapidity of viral movement in peripheral nerves, movement through the CNS, and peripheral dissemination of virus. Following development of immunofluorescent and electron microscopic techniques, finer details of viral transit and virus-cell interactions were revealed. Use of recently developed monoclonal antibody technology, protein analysis, molecular biology and studies of cellular receptors has already revolutionized recently held concepts of the nature of "street virus", and begun to provide reasons for cellular specificity and neurotropism of the agent. This review, including some previously unpublished data, is an attempt to present a concise view of the pathogenesis of rabies, and to point out areas requiring further research.

Probably because of convenience and the large amount of existing data (physiologic, anatomic and immunologic) on mice and rats, laboratory rodents have been used for most of the pathogenetic studies done during the past several years. Although there are species similarities rendering many of the results in rodent experiments valid when extrapolated to other animals, there are also species differences in reaction

to rabies. It is thus appropriate that more emphasis be placed on animals important in the naturally occurring disease.

A general scheme of the pathogenesis of rabies includes the following steps in movement of virus through the animal body: (i) introduction of virus into a bite wound or laceration (less commonly through mucous membranes); (ii) migration via peripheral nerves to the CNS; (iii) spread through the CNS; (iv) centrifugal neural transport of virus, and (v) infection of non-nervous tissues. This general concept of the "flow" of infection is well substantiated and widely accepted. Early experimental work involved animal inoculation and subsequent isolation of virus from many tissues and organs. For details of early studies see reviews in references 1-4. Recent and current research is centered on details of the infectious process at various sites and stages of the disease. Obviously the pathogenesis includes all the interactions of host and infectious agent. To a large extent this means the sequential infection of cells including binding of virus to cellular receptors, endocytosis, translation and replication of viral RNA, assembly of virions, and cellular release of progeny virus. Where appropriate some of these features will be mentioned, but for a detailed description of virus-cell interactions see Tsiang (this volume).

ENTRANCE OF VIRUS INTO THE ANIMAL BODY

Many historic descriptions (5) and current research on rabies indicate that the naturally occurring disease is generally transmitted by bites of rabid animals. The disease was experimentally transmitted by saliva from rabid animals early in the 19th century, first by Zinke in 1804 (6) and later by others (7,8). Viral entrance through mucous membranes, though much less frequent than bite transmission, may be important in environments conducive to airborne infection, as in bat caves (9,10), or in the initiation of epizootic rabies by consumption of frozen carcasses of rabid animals in the Arctic (11-14).

Inoculation Site (Bite Wound or Laceration)

One of the primary concerns with the inoculation site is whether virus enters peripheral nerves directly (without preliminary replica-

tion in non-nervous tissue), or indirectly (following replication in non-nervous tissue). Postexposure treatment (immune serum, vaccine, wound cleansing, etc.) in man is usually given as soon as possible after contact with a rabid animal. This is based on the assumption that removal or neutralization of virus before entry into peripheral nerves is likely to be much more successful than similar measures attempted after viral entry into the nervous system. It is mainly within this context that events at the inoculation site are important. Experimental studies supporting direct entrance into peripheral nerves indicate that in many cases, virus leaves the inoculation site and is on its way to the CNS within a few hrs of inoculation. Such studies have used nerve resection or limb amputation proximal to the site of inoculation of rabies virus at various times before or after inoculation. Using such methods in mice, it was demonstrated that Challenge Virus Standard (CVS) rabies virus could leave the inoculation site (foot pad) by 4 hr post-inoculation (15). Amputation of the inoculated foot at 8 hr had no sparing effect (15). In rabbits inoculated intramuscularly (IM) with "Fermi" virus, virus had left the inoculation site by 8 hr (4). Baer *et al.* (16) neurectomized rats at various times after challenge (foot pad inoculation) and found that, in many cases, CVS virus left the inoculation site by 8-10 hr. Baer *et al.* (17) stated that the length of time after which operative procedures (in rats inoculated with street virus) were no longer effective appeared to vary considerably. For some it was only 24 hr, yet for others operative procedures were effective more than 96 hr after foot pad inoculation. In mice inoculated with a suspension of salivary gland from a rabid bobcat (characterized by long incubation periods), a few deaths occurred even when amputation of the inoculated limb was done 24 hr after inoculation (18).

The above studies indicate that in some cases the delay between inoculation and departure of virus from the inoculation site is insufficient for replication in and release from non-nervous cells. Replication of fixed virus in tissue culture usually requires 12-15 hr for virus to appear in the medium (19). Iwasaki (20) found the earliest time of release to be 6 hr. As discussed later, animals (hamsters, skunks) inoculated with fixed or street virus had immuno-

fluorescence in muscle cells at the inoculation site much later (hamsters, 36 hr; skunks, 7 days) than the above times at which neurectomy was not life saving for all the animals (21,22). These features strongly support the contention that in some cases rabies virus enters peripheral nerves directly without preliminary replication in non-nervous tissue.

Recent studies suggested a role for the acetylcholine receptor (AChR) in uptake of rabies virus by axons of motor neurons. Watson *et al.* (23) reported that mice inoculated intramuscularly (IM) with CVS virus, had immunofluorescent foci in inoculated muscle (in the endomysium) at 1 hr post-inoculation, and that these foci were similar in form and distribution to neuromuscular junctions (NMJ) determined by acetylcholinesterase staining. Binding to AChRs would seem to offer an excellent mechanism for retention at the NMJ. However, since there is no known chemotaxis to account for movement of virions in interstitial spaces, binding alone does not explain absence of virions in areas of the endomysium remote from NMJs at 1 hr post-inoculation. In skunks (*Mephitis mephitis*) inoculated IM with street virus, antigen was present in wide and thin bands in the endomysium (22). There was no localization to foci consistent with NMJs.

Support for a role for AChRs came from studies demonstrating colorization of rabies virus and high density areas of AChR at NMJ on chick myotubes, and suppression of infection of chick myotubes, by application of alpha-bungarotoxin and d-tubocurarine (24-26). Lentz *et al.* (27) determined a similarity in amino acid sequence of rabies virus (CVS and ERA strains) glycoprotein and snake venom curaremimetic neurotoxins. Binding of rabies virus to AChR of *Torpedo californica* was maximum at a low pH (5.5) of the incubation medium (28). It has been suggested that AChRs serve to concentrate virus at NMJs, thereby facilitating uptake by peripheral nerves (26). The precise mechanism involved in this direct entry into peripheral nerve (binding to and release of virus from AChR on myocyte plasma membrane and/or binding of virus to receptors (?) on the axolemma of the axon terminal) has not been determined. Alternatively, binding to myocyte AChR could induce endocytosis by muscle cells (28) providing for indirect entry into nerves as discussed later.

The evidence to date suggests very strongly that rabies virus can bind to the acetylcholine receptor, but this does not seem to be the only membrane component facilitating cellular uptake of the virus. Infection occurs in several cell lines that do not have acetylcholine receptors (29). Tsiang (30) states that the rabies receptor probably is not a single molecule but is likely a complex of components in the plasma membrane. There is evidence for binding of rabies virus to the following components of cell membranes: neuraminic acid (31), carbohydrate moieties (32), and phospholipids (including phosphatidyl serine and gangliosides) (33-37). Possibly the cellular receptors for rabies are subject to the vagaries of viral strain and other factors, as has been demonstrated with other viruses. Some cardiovirus variants may utilize different receptors on the same cell and cellular receptors may be modulated by cell growth and differentiation (38-40). As is well known, rabies street virus differs markedly from CVS virus in the time of arrival in the CNS after peripheral inoculation. This may be related, at least in part, to interaction of virus and cellular receptors. Possibly, as suggested by Tsiang (this volume), the binding of rabies virus to cells at the inoculation site is, at least partly, dependent on transient conditions of the microenvironment that may be extremely difficult to determine and to duplicate *in vitro*.

The question of indirect entry of virus into peripheral nerves should be considered from two aspects. First, infection of non-nervous tissue at the inoculation site and, second, evidence that this infection actually can be an intermediate step or link in the pathogenesis.

Virus isolated from the inoculation site for a short period (usually 24 hr or less) after inoculation (17,41-45) probably represents virus in the inoculum (before entrance into any cells). Isolations at later times (after CNS infection) (42-43,46-47) may be the result of centrifugal migration of virus and, thus, not an accurate indication of early events at the inoculation site. Murphy and coworkers (21) were the first to demonstrate infection of myocytes (by immunofluorescence and electron microscopy) at the inoculation site. Immunofluorescence was detected in striated muscle cells in hamsters infected with all the viruses studied (Lagos bat, vampire bat, CVS and Arctic fox rabies viruses). Antigen was first detected in myocytes at

36 hr post-inoculation. Charlton and Casey (22) demonstrated infection of myocytes at the inoculation site of skunks inoculated with a skunk street virus (a subsequent study demonstrated that the muscle fibers were infected directly by virus in the inoculum) (48). During the first 24 hr, immunofluorescence of the inoculum was detected in the endomysium in bands of variable thickness; foci compatible with localization of virus at NMJ were not seen. Antigen was detected in muscle fibers first at 7 days post-inoculation, before detection of antigen in the CNS. Budding on the sarcolemma, as noted by Murphy *et al.* (21) in hamsters, was not detected and infectious virus could not be isolated after 24 hr (22). Tsiang *et al.* (49) found that cultured primary rat myotubes were susceptible to rabies virus (CVS and street virus) infection. Street virus but not CVS virus produced infective viral particles. They suggested that, for CVS virus, entrance into peripheral nerves was direct rather than indirect through myocytes.

The above studies demonstrate conclusively that infection of myocytes can occur at the inoculation site. These same studies do not demonstrate that indirect entrance into peripheral nerves, via myocytes, is an essential link in the pathogenesis; i.e., that in some cases the indirect route occurs to the exclusion of the direct route. Because of the transit time for virus to travel to the CNS and the marked variations in incubation periods due simply to differing doses given intracerebrally (IC), it is quite possible to have direct viral entry into peripheral nerves and still have immunofluorescence in muscle fibers preceding any detected in the CNS. A report by Baer and Cleary (18) would seem to be the only evidence indicating that indirect entry can occur to the exclusion of direct entry. These scientists demonstrated that street virus (bobcat isolate) could be retained at the inoculation site in mice at least until 18 days post-inoculation (amputation of inoculated foot at 18 days was life saving for most of the mice). While this did not demonstrate the tissue involved, it is fairly certain that replication must have occurred in extraneural tissues. This area requires additional investigation to firmly establish indirect entry into peripheral nerve as a mechanism in animals important in the naturally occurring disease.

Because of interest in the possibility that virus is retained in muscle at the inoculation site during long incubation periods, we studied this feature by denervation of the abductor digiti quinti (ADQ) muscle before inoculation in skunks. In this muscle, antigen was in muscle fibers at 7 days and remained until 28 days post-inoculation but not beyond (48). Thus, infected muscle could be a source of virus (for transit in peripheral nerves) for moderate incubation periods. However, the findings did not support the contention that the long incubation periods are due to long term retention of virus in myocytes. Virus was considered to be retained at the inoculation site (in muscle) of experimentally infected marmots for at least 35 days (50). Further studies should determine if avoidance or suppression of the immune response would prolong myocyte infection.

There is some evidence that street virus enzootic in Ontario skunks is more infective for skunk skeletal muscle than CVS virus or some vaccine virus strains (51). Groups of 4 skunks were inoculated intramuscularly (right ADQ) with 0.3 ml of the following strains: skunk street virus (10^{-1} and 10^{-4} dilutions of submandibular salivary gland suspension); CVS virus (10^{-1} and 10^{-4} dilutions of mouse brain suspension); Flury Low Egg Passage (LEP) vaccine (Fromm Laboratories); Flury High Egg Passage (HEP) vaccine (Norden Laboratories); ERA^R vaccine (Connaught Laboratories); and Trimune (Fort Dodge Laboratories) (Table 1). All the skunks were killed on day 16 and sections of right ADQ were examined for rabies immunofluorescence. Skunks given low or high doses of street virus regularly developed myocyte infection (as measured by immunofluorescence) in the inoculated muscle, whereas with CVS virus, only high doses resulted in myocyte infection. Of the vaccine strains, only HEP infected myocytes, and this occurred in only 1 of 4 skunks (Table 1).

This brief study suggests that Ontario skunk street virus has greater infectivity for skunk muscle than any of the other strains tested. The live vaccines (LEP, HEP and ERA^R), generally considered to replicate in the vaccinated animal, do not readily infect and grow in skunk skeletal muscle. This should not be interpreted as necessarily supporting the indirect route; the results may only reflect adaptation of the skunk street virus to skunk tissue. As mentioned above, a

Table 1. Infectivity of Rabies Viral Strains for Skunk Skeletal Muscle

Virus strain	Titer[a] of virus inoculated[b]	Positive skunks[c]/ no. inoculated
Skunk street	4.3	4/4
	1.3	4/4
CVS	4.8	3/4
	1.8	0/4
Flury LEP	3.7	0/4
Flury HEP	0[d]	1/4
ERA^R	3.3	0/4
Trimune[e]	0	0/4

[a] $MICLD_{50}$ (mouse intracerebral 50% lethal dose)/0.03 ml inoculum.

[b] Site of inoculation: right ADQ muscle.

[c] Number of skunks with rabies immunofluorescence in myocytes of the ADQ muscle on day 16.

[d] Flury HEP is not lethal in weanling and adult mice.

[e] Inactivated vaccine.

street virus isolate (European fox, *Vulpes vulpes*) induced a much more productive infection in cultured rat myotubes than a fixed strain (CVS) (49).

One would expect many torn and severed nerve fibers in bite wounds inflicted by terrestrial vectors of rabies. Whether or not uptake of virus by cut ends of nerves is an important factor in the pathogenesis has not been reported. Exposure of cut ends of rat sciatic nerve to rabies virus did not produce high morbidity rates (16). Experimentally there is uptake of horseradish peroxidase from cut ends of the vagus nerve and centripetal axonal migration (52). In our laboratory, skunks inoculated IM (intramasseter route) had markedly shorter incubation periods if the muscle was exposed surgically for inoculation than if inoculation was made through intact skin and into the muscle (53). The experiments were not designed to study this phenomenon and, although the doses (in $MICLD_{50}$) were closely similar, different virus prepara-

tions were used. Further studies should be undertaken to determine if there is viral uptake by severed nerves and/or if other effects of trauma influence the incubation period.

Viral Entrance through Mucosal Surfaces

Although infection via mucosae probably is infrequent, it may be important in special environments. Occasional spread by oral transmission could help to maintain or initiate enzootic rabies. Incidents suggestive of oral or nasal infection include human infection by airborne infection in bat caves (10), possibly by aerosol from wolves (1), and infection of dogs by consumption of rabid fox carcasses in the Arctic (54). Although virus was isolated from mammary glands of infected gravid bats, conclusive evidence of transmission to nursing bats via milk could not be obtained (55). Experimentally, rabies has been transmitted via the oral route to mice (56-64), rats (60), hamsters, guinea pigs and rabbits (59,64), foxes (*Vulpes vulpes*) (65-68), skunks (69,70), and cats (68). In many of the above studies, virus was administered by unnatural methods such as forced feeding with a syringe or other dispenser. In studies in white Swiss mice and skunks given very large doses of CVS or street virus free choice, none of the skunks and only a very small percentage of mice developed rabies (56,71). Serokowa (61) suggested that, under natural conditions infection after eating large amounts of infected material probably occurs by the transnasal route through the nasopharynx or by external contamination of the nostrils. The precise site(s) or mechanism of viral penetration has not been established for oral infection. An important sequel to the demonstration of oral infection was the development of oral rabies vaccines for wildlife. Studies conducted in the United States, Canada, and Europe demonstrate that foxes can be readily immunized via absorption of conventional live vaccine virus through the oral mucosa (65,72,73), whereas skunks are generally refractory to the same vaccines given orally (W.G. Winkler, personal communication) (74). Since vaccination of foxes against rabies by the oral route has been very successful in Switzerland (see Wandeler, this volume), the practice is likely to become much more widespread and will include other species. For development of vaccines and baits (including adjuvants), it will thus be important to determine the precise

site(s) and mechanism of absorption of virus. It has been suggested that in foxes vaccine virus first localized in the tonsils (75).

Some authors have concluded that infection can occur through the intestinal mucosa (57). A live vaccine administered into the intestine of foxes induced an immune response (76). Although CVS virus administered directly into the intestine of skunks induced neither disease nor an immune response (71), similar experiments with an experimental recombinant vaccine induced immune responses and protection against challenge in several skunks (74). There are conflicting reports regarding rectal instillation of rabies virus. Reagan *et al.* (77) produced rabies in hamsters by this route, but similar experiments by Robertson and Beauregard (78) failed to produce infection.

Experimentally, the intranasal route has been demonstrated in mice (56,58,61,79-82), guinea pigs (80,83,84), hamsters (21), rabbits (80), bats (85), and skunks (71). Probably aerosol exposure was responsible for an outbreak of rabies in a laboratory colony of foxes (86). In weanling mice (CD1) given CVS or a skunk street virus, one intranasal lethal dose 50 ($INLD_{50}$) was equal to approximately 10^3 $MICLD_{50}$ (56).

It is widely accepted that the route to the brain after intranasal exposure is via the olfactory epithelial cells in the olfactory mucosa (87). The olfactory epithelial cell is a bipolar neuron with the cell body in the olfactory mucosa, one process extending to the surface of the epithelium and the other extending to the olfactory bulb. This viral route to the brain might, in large part, allow viral entrance without neutralization by components of the immune response. Using the fluorescent antibody technique, antigen was detected in cells of the nasal mucosa of naturally infected bats (88), and in mucosal epithelial cells and nerve cells in the submucosa of mice inoculated intranasally (58). Hronovsky and Benda (80) found virus in mucosal epithelial cells one day earlier than in the brain and it was assumed that virus travelled centripetally along olfactory and/or trigeminal nerves. In unpublished studies we found that olfactory bulbectomy did not prevent development of rabies in many mice exposed intranasally to CVS rabies virus. This suggests that the olfactory route is not essential for transit of virus to the brain. Fibers of the trigeminal nerve ramify

in the nasal mucosa and may be an alternative route to the brain. Berry and Slavin (89) found that after intranasal infection of mice with herpes virus, the agent was first found in the nasal mucosa from which it progressed to the gasserian and superior cervical ganglia, and then to the brain.

Rabies following application of virus to the abraded cornea probably indicates uptake of rabies virus by sensory terminals since the cornea is well innervated by sensory fibers and has no known motor innervation. This mode of infection using fixed and street virus in rabbits was reported by Centanni and Muzzio (90). Exposure of intact conjunctiva resulted in deaths of 1 of 7 rabbits, whereas all of 102 rabbits exposed via the abraded cornea succumbed (90). Selective uptake by nerves to the eye and routes to the brain were demonstrated in some excellent studies by Kucera *et al.* (91). The primary neural sites of uptake of CVS rabies virus after inoculation of the anterior chamber were intraocular parasympathetic (muscarinic cholinergic) oculomotor fibers, retinopetal fibers, and fibers of the ophthalmic nerve (branch of the trigeminal). There was no uptake by the optic nerve or by adrenergic fibers (from the superior cervical ganglion). An apathogenic variant of CVS virus penetrated the ophthalmic nerve as evidenced by antigen in the trigeminal ganglion at 48-58 hrs, but there was no uptake by the oculomotor or retinopetal fibers. Infected neurons in the CNS were never extensive (91) after infection by the apathogenic variant.

ROUTE TO THE CNS

Early localized paralysis and lesions in the CNS associated with entrance of nerves from the inoculation site suggested centripetal spread along peripheral nerves (92,93). More definitive evidence for the neural route to the CNS was as follows: neurectomy proximal to the inoculation site before or shortly after inoculation of rabies virus and amputation soon after inoculation generally prevented development of clinical disease (4,15-18,94-99). After peripheral inoculation in the intact animal, antigen and infective virus occurred first in the region of the CNS having neural connections to the inoculation site

(17,41,44,100-104). Substances that impair nerve function can diminish the incidence of rabies if applied proximal to the inoculation site (15,105,106). Additional observations that support peripheral nerve involvement include paresthesias that develop in human rabies (107) and self mutilation in experimental rabies in foxes in regions frequently referable to the inoculation site (108,109).

The above studies have included several species and demonstrate conclusively that the principal pathway to the CNS is via peripheral nerves. They do not indicate the precise part of the nerve within which viral transit occurs. Further resolution of this problem was provided by Dean *et al.* (15). Their experiments demonstrated that fixed virus could move along peripheral nerves at approximately 3 mm/hr, a rate considered to be too fast to be attributed to cycles of replication and cell-to-cell (in Schwann cells or fibrocytes) transfer along peripheral nerve trunks and thus indicative of transit of virus in a fluid or semifluid medium. Schneider (44) also deduced that virus was carried passively.

General possibilities for passive transport of various substances in peripheral nerves were reviewed by Kristenson and Olsson (110). They include axoplasm of peripheral nerves, and fluids in the endoneural, subperineural and epineural spaces. The subperineural space is continuous with the intrafascicular (endoneural, interstitial) spaces of the nerve fascicle. There are no perineural spaces as such and epineural spaces refers to interstitial spaces in the epineurium (111). Lymphatics are present only in the epineurium, do not constitute direct channels to the CNS (111) and, therefore, are unlikely conduits of virus to the CNS. Kristenson and Olsson (110) stated that because of the structural relationships of epineurium and perineurium with meninges, it seems possible that tracers injected into epineural or subperineural spaces could reach the subarachnoid space. Radiographic contrast media and horseradish peroxidase injected into the endoneurium will spread widely in the intercellular spaces around fibers and in subperineural spaces, eventually reaching the CNS. However, in studies employing intramuscular inoculation of tracers this route was discounted for the following reasons: (i) no tracer was detected in the neuropil surrounding tracer-containing neurons; (ii)

tracer was not detected in the endoneurium; and (iii) axonal destruction by crushing or interference with axoplasmic flow by colchicine prevented transfer to neuronal perikarya (110).

When the mitotic inhibitors colchicine and vinblastine were applied to peripheral nerves proximal to the site of inoculation of rabies virus, clinical rabies did not occur (112,113). This established axoplasm as the conduit and retrograde axoplasmic flow as the mechanism of viral transport to the CNS. The rate of movement is similar to that described for poliomyelitis virus and for some proteins given experimentally. Additional evidence supporting the intra-axonal route includes general absence (with a few exceptions) of virions or matrix in Schwann cells or endoneural fibrocytes, even late in the disease.

In the peripheral nervous system (except for short dendrites within autonomic ganglia) peripheral neural processes are axons and thus generally devoid of granular endoplasmic reticulum and free ribosomes (114-117). This would seem to obviate intra-axonal viral replication while virus is en route to the CNS. Some previous reports contain illustrations depicting viral matrix and virions in axons (21,81). The tissues were taken at times after occurrence of viral antigen in spinal cord or cerebrospinal ganglia and may well represent centrifugal migration of these structures. This aspect is discussed further under centrifugal dissemination of virus.

Nerve Fibers Involved in Transport

A few studies suggest that rabies virus can migrate to the CNS in either motor or sensory fibers. After inoculation of the rear foot pad of mice with a fixed virus, Schneider found immunofluorescence first in neurons of ipsilateral dorsal root ganglia (44) suggesting migration via sensory fibers. Others found that immunofluorescence occurred in dorsal root ganglia and spinal cord simultaneously (41). Infection of rabbits via the scarified cornea (90) supports the sensory route since the innervation of the cornea is entirely sensory. As mentioned above, virus may migrate centripetally in sensory fibers of the trigeminal nerve, and in oculomotor (parasympathetic) and retinopetal fibers (90). Recent studies of the role of acetylcholine receptors suggest transit by motor fibers (23,26). Dean *et al.* (15) attempted to

determine whether viral transit occurred by motor or sensory fibers or both. Prior to inoculating CVS virus into the foot pads of mice, the dorsal or ventral roots of spinal nerves from the 5th lumbar nerve caudally were sectioned. Rabies developed in mice subjected to either surgical procedure. However, the study did not take into account connections with the abdominal part of the sympathetic trunk, and viral movement in autonomic fibers could not be ruled out.

Blood-borne Infection

Although peripheral nerves constitute the main pathway to the CNS, there is experimental evidence that virus may occasionally take a blood-vascular route in some species (118). For a review of early studies, see Eichwald and Pitzschke (2). Pasteur produced rabies in dogs by inoculation of virus into the blood stream (119). He considered that the spinal cord was affected first and that infection by this route was more likely to produce dumb than furious rabies. Others have produced rabies by this route in mice (15,44,58) and guinea pigs (120). Dean *et al.* (15) found that in neurectomized mice and rats given virus into the foot pad, 86.7% of 13 day-old mice and 60% of 19 day-old rats died of rabies whereas older animals generally survived. Also the sparing effect of neurectomy in 60-100 g hamsters varied with the dose of virus. Dean (121) stated that exceptions to the neural route include young animals, animals of very susceptible species such as the hamster, and those in which resistance has been altered as by intracerebral trauma or shock. This would, then be a factor to consider in certain types of experimental studies. It would seem that the hamster and very young mice and rats are not ideal experimental animals for study of neural migration of virus to the CNS.

Site of Exposure

It is generally conceded that bites inflicted to the head and neck of humans are much more likely to result in clinical disease and that incubation periods are shorter than those following bites to the extremities (122). Dean *et al.* (123) reported that guinea pigs exposed to fixed virus via a hole in the skin of the neck developed rabies whereas those exposed in the same manner in the back leg survived. Baer (118), citing Parker, stated that foxes were much more susceptible to low egg passage Flury virus via the neck muscles than muscles of the

rear leg. Others have noted marked species variation in susceptibility by different routes (109). In dogs, inoculation in the hind paw prolonged the incubation period compared to inoculation of head muscles (109). Cats were especially refractory (low frequency of clinical disease) to inoculation into the masseter muscles (109, K. Lawson, personal communication), but inoculation into cervical muscles was wholly satisfactory (109). As described above we have had similar findings in skunks in that intramasseter inoculation (without surgical exposure) produced much longer incubation periods than intramasseter inoculation (with surgical exposure).

Light Microscopic Lesions in Dorsal Root Ganglia and Peripheral Nerve

As previously discussed, evidence to date strongly suggests that rabies viral transit to the CNS can occur via motor or sensory fibers. With motor fibers the first cell bodies encountered are those of the ventral motor neurons or those in autonomic ganglia; with sensory fibers, the cell bodies are in dorsal root or cranial sensory ganglia. According to current dogma, these perikarya are the first sites in the nervous system where viral replication can occur. Dorsal root neurons are unipolar. The single axon bifurcates a short distance from the cell body into a central and a peripheral process. It is not known whether or not it is essential for rabies virus to replicate in the dorsal root perikaryon or whether virus may travel directly from the peripheral to central process without entering the cell body. Virus that reaches the CNS via the central processes of the neurons supposedly could directly infect neurons in several adjacent segments of the spinal cord (brain stem in exposures of the head) or, in some cases travel long distances in a single axon to its termination in the nucleus gracilis or nucleus cuneatus in the medulla oblongata. Apparently long distance migration (e.g., from neuron(s) in lumbar dorsal root ganglia to the nucleus gracilis or cuneatus) is uncommon since most studies of the sequential development of antigen describe first occurrence of antigen in segments of the spinal cord with direct linkage to the inoculation site.

During centrifugal dissemination of virus from the CNS (that occurs both early and late in the disease process) there is infection of perikarya of dorsal root ganglia neurons, resulting terminally in

widespread involvement of ganglia, many cells containing antigen. Usually at this stage there is moderate to severe inflammatory response (lymphocytes, plasma cells and macrophages) in ganglia and, unlike the CNS, there is extensive neuronal degeneration (92,124). The neuronal degeneration apparently is not dependent on the immune response (124). In skunks the changes of neuronal degeneration included increased cytoplasmic eosinophilia, central chromatolysis, complete chromatolysis, cytoplasmic vacuolation, nuclear pyknosis and karyorrhexis, or replacement of the cell body by macrophages. At one time (especially before discovery of Negri bodies), histologic examination of gasserian and dorsal root ganglia was useful in rabies diagnosis (125). In peripheral nerves, there may be regional and focal accumulations of mononuclear cells and Wallerian degeneration of scattered fibers (22,126,127). The Wallerian degeneration is probably the result of neuronal degeneration in dorsal root ganglia. Segmental demyelination and remyelination and, to a lesser extent, Wallerian degeneration have been reported in cases of paralytic rabies in man (128). In classical human rabies, there are neuronal degeneration in dorsal root ganglia, proliferation of capsular cells, leucocytic accumulations (in nerves) and axonal swelling (129). This area deserves further investigation since only one (128) of the above studies used teased fibers to characterize the lesions.

THE CENTRAL NERVOUS SYSTEM

Replication of rabies virus in the CNS occurs mainly in neurons. Spread of infection is considered to be cell-to-cell (transneuronal) eventually resulting in extensive dissemination of antigen in the brain and spinal cord. Centrifugal spread in peripheral nerves occurs simultaneously and accounts for peripheral spread of virus and infection of several non-nervous tissues.

Studies of the sequential development of the disease by tissue titration and/or immunofluorescence suggest that infection generally spreads in a spatially integrated fashion (with infection spreading stepwise to adjacent areas) from the entry site in the CNS to most areas of the brain and spinal cord (16,42,82,85,100,101,130,131). However spread can be extremely rapid (41,45,131); it would seem likely

that neurons with long axons would provide a means of 'leap-frogging' of infection to distant areas. Schneider (132) estimated that in small laboratory animals 70-80 hrs are required for spread of street virus through the CNS but only 24 hrs for fixed virus. There is little information on the time required for spread in the spinal cord and brain in domestic and wild animals. In most naturally occurring cases of rabies, antigen is widespread in both the brain and spinal cord at the time of euthanasia or death. Although widely disseminated there are fairly marked regional differences in intensity of staining (number of neurons affected and amount of antigen per neuron) by immunocytochemical methods. Experimentally, animals inoculated in sites remote from the head generally have antigen in both the brain and spinal cord at the onset of clinical signs. Cases in which the infection is apparently confined to the spinal cord have been reported (133) but probably are infrequent. A horse euthanized because of paralysis had antigen in the lumbar spinal cord but not in the hippocampus (133). Since other areas of the brain were not examined, it is not conclusive that antigen was restricted to spinal cord. There may be fairly marked differences in antigen concentration in various parts of the brain in rabid cats (134). For diagnosis of field cases, routine testing of spinal cord, (in addition to brain), is not recommended but should be considered in animals with unusual or prolonged clinical signs.

Johnson (41) described early selectivity for neurons of the limbic system and relative sparing of the neocortex - with the progression of clinical signs; i.e., initial alertness and aggressiveness without seizures and motor dysfunction (85). Aggressive behavior is a very complex neural function involving several areas of the brain (135,136). Determination of the mechanism in rabies will require (for a beginning) precise delineation of severely and slightly affected neuronal populations and studies of neurophysiologic abnormalities. Since many of the above studies on selective vulnerability were done with fixed virus, usually in small laboratory animals, it is appropriate to investigate these features of the disease using recently developed immunocytochemical methods on tissues from known rabies vectors infected with street virus. Although antigen is widespread in rabid skunk brain, some regions fairly consistently contain high concentrations. These areas

include the midbrain raphe nuclei, hypothalamus, hippocampus, nucleus locus ceruleus and scattered large neurons in the reticular formation. Other areas vary in amount of antigen (heavy or light). Although the study is only in initial stages, it is of interest that the midbrain raphe nuclei and the medial hypothalamus are inhibitory centers of aggressive behavior (135,137).

In CVS-infected mouse brain, antigen as detected by immunofluorescence occurred only in neurons, not in glial, ependymal or endothelial cells (138). Schneider (132) found only slight involvement of meninges, ependymal cells, and glial cells. Likewise Murphy *et al.* (131) found only a few meningeal cells and occasional ependymal foci containing antigen. In skunks infected with street virus we have never found antigen (PAP method) in meningeal cells. Antigen occurred very rarely in ependymal cells. However in late stages of the disease we detected several oligodendrocytes that contained antigen. They were most readily detected in the corpus callosum and white matter of the cerebral cortex (108). In an *in vitro* study, neurons were infected much more readily than glial cells (139). In neurons, dendrites generally contain much more antigen than axons. This is probably due to the distribution of ribosomes in neurons, i.e. presence in perikarya and dendrites and absence in axons (22).

Electronmicroscopic findings generally confirm the predominance of replication to neurons. In 1962, Roots (140) and Matsumoto (141) reported EM findings of rabies virus *in vivo*. Almeida *et al.* (142) described the ultrastructure of rabies virus in tissue culture. Since then several scientists have published reports on the ultrastructural features of rabies virus replication in nervous tissue (22,81,131,138, 143-156). The morphogenesis of rabies virus occurs in the neuronal cytoplasm; the nucleus does not contain antigen or virus (see Tordo (this volume) regarding possible migration of leader RNA to the nucleus). In early neuronal infection, small accumulations of matrix and virions frequently occur adjacent to Nissl granules, suggesting that replication begins in these areas (157). This feature is more readily observed in neurons of dorsal root ganglia since in these cells the Nissl bodies are fairly clearly demarcated and separated by abundant neurofilaments. Structures that have been considered to be

products of the replicative process include viral matrix, mature virions, convoluted membranous profiles, bizarre tubular structures, and dense-granular foci. These structures can occur singly or in any combination. Matrix may occur in small to fairly large bodies of finely granular or filamentous material. It consists of randomly oriented strands of viral nucleocapsid (158). For excellent illustrations of this filamentous material in neurons, see Murphy *et al.* (131). During the course of infection, there is progressive condensation of strands and increase in number, size, and complexity of matrices in neurons (159). The bodies of matrix and other rabies structures usually appear in the neuronal cytoplasm with no space separating them from the surrounding cellular organelles. The convoluted membranous profiles and bizarre tubular structures probably represent aberrant or anomalous viral morphogenesis. In fixed virus infections bodies of matrix are generally small and mature virus particles are sparse. In street virus infections there is a wide range in size of matrices, with many large bodies. Generally virus particles are more numerous in street than in fixed virus infections in brains of mice (159). Since fixed virus occurs at higher titer (in brain) than street virus, the efficiency of morphogenesis in the former must exceed that of the latter; i.e., many of the virus particles in brain infected with street virus are noninfectious. Tsiang and Guillon (160) found that glycoprotein was not detected (by immunofluorescence) in brains of mice infected with street virus but was in those infected with CVS virus, suggesting less glycoprotein synthesis in street infections. Animals with naturally occurring or experimental street virus infections frequently have titers of virus in submandibular salivary glands that are 100- to 1000-fold higher than those in brain, suggesting that replication or efficiency of replication in salivary gland exceeds that in brain.

Small bodies of matrix without entrapped cellular organelles are considered to be Lyssa bodies. Light microscopically, Lyssa bodies are small, homogeneous intracytoplasmic bodies that lack the internal structure of Negri bodies (92). Inclusions (Negri bodies) were described first by Babes (1). He stated that since they (the inclusions) were not present in all cases of rabies, he did not attribute to

them all the importance they deserved (1). Matsumoto (161) reviewed the historical considerations of the Negri body and described the ultrastructural features. The internal structure is a cardinal feature of the light microscopic appearance and has been attributed to cellular organelles and virions trapped within the body of matrix. Probably ribosomes are more important for this feature than other organelles and virions since they readily stain with basophilic dyes. The various products of viral replication occur in perikarya and dendrites and, to a lesser extent, in axons. Their presence in axons is probably the result of cellulifugal (i.e., from the cell body) transport or centripetal transport from the axon terminal (after transneuronal dendroaxonal transfer of virions) since axons are generally devoid of ribosomes.

Viral budding occurs on many membranes in the cell including the rough and smooth endoplasmic reticulum, the outer nuclear membrane and neurotubules. Apparently modification and eversion of host cell membrane occurs synchronously with coiling and attachment of nucleocapsid to the everting plasma membrane (159). After coiling is complete, the virus particle pinches off (159). During early ultrastructural studies, there was little or no evidence of budding on neuronal plasma membranes *in vivo*, but virus assembly was demonstrated on plasma membranes of infected chick embryo fibroblasts and BHK-21 cells (159,162-167). Studies by Iwasaki and Clark (168) and Charlton and Casey (22) demonstrated budding on the neuronal plasma membrane in brains of mice and skunks. Budding occurred on the perikaryal and dendritic plasma membrane, including the postsynaptic membrane (22). There was simultaneous esotropic uptake of virus by adjacent axon terminals. In all cases so far examined in skunks, budding was directed opposite to the polarity of the synapse. This dendroaxonal transfer of virus was considered to be dependent on sites of viral protein synthesis (dendrites and perikarya) and this in turn on the distribution of ribosomes. Ribosomes are abundant in perikarya and dendrites, but generally absent from axons (except for small numbers in the axon hillock). After viral budding (on dendritic or perikaryal plasma membrane) and esotropy by the adjacent axon terminal, the virion is transported by retrograde axoplasmic flow to the cell body and dendrites of the recipient neuron. This retrograde movement is

supported by the work of Tsiang and coworkers. Stereotaxic inoculation of CVS rabies virus into the striatum of rats was followed by retrograde axoplasmic transport of virions to perikarya in the substantia nigra (169). Inoculation of colchicine into the striatum prior to inoculation of virus impeded transport of virions to neuronal perikarya (P.-E. Ceccaldi, personal communication). Dissemination of infection is considered to involve successive cycles of replication, transneuronal transfer of virus, and retrograde axonal transport.

In examination of thin sections of grey matter by electron microscopy, the number of intracellular virions usually far exceeds the number of virions budding on plasma membrane. This disparity had led some researchers to conclude that dendroaxonal transfer of virus is relatively unimportant in dissemination of infection of the CNS. However, the amount of neuronal plasma membrane actually examined in one thin section by transmission electron microscopy is probably less than 1/1000th of the plasma membrane of a single large neuron with extensive processes, suggesting that a few budding viruses (on plasma membrane) are highly significant in regard to transneuronal transfer. Since neuronal degeneration is much less extensive than neuronal infection, release of intracellular virions through disruption of necrotic neurons is not likely to be an important mechanism in dissemination of infection.

As reviewed by Schneider (102), the cerebrospinal fluid (CSF) has been suggested as a transport vehicle mediating rapid dissemination of virus to many parts of the CNS. However, isolations of virus from CSF have been infrequent and mainly at late stages of the disease (2,102). Also, there is little evidence of infection of ependymal or pial cells even though antigen may be present in adjacent grey matter. Although this proposed mechanism deserves further study, it is unlikely to be important in the pathogenesis. Blood-borne infection was discussed under routes to the CNS. It is not considered to be important except in some highly susceptible species.

Inflammatory Lesions

The polioencephalomyelitis of rabies is characterized by perivascular cuffing with mononuclear cells, focal and regional gliosis, and neuronophagia. Lesions occur in most areas of the CNS but freque-

ntly are more severe in or are restricted to the brain stem. Schulz (170) noted that several rabid cattle had non-purulent encephalitis confined to the medulla oblongata. Meningitis is usually present to some degree. The essential features were recognized very early in the application of histopathologic techniques to the study of rabies (171-175) and since then, many other authors have confirmed and elaborated on the light microscopic features of the disease (152). Although the lesions vary in severity they are present in most rabid animals. A few rabid animals have brains virtually devoid of inflammatory lesions. In our experience this occurs more frequently in cattle than in other species. In experimental rabies in skunks given street or CVS virus, less than 1% had brain without inflammatory lesions (unpublished observations).

Perivascular cuffs contain lymphocytes, plasma cells, macrophages and, occasionally, erythrocytes. The number of plasma cells generally increases with increasing duration of clinical signs. In many of our experimental skunks there are several scattered plasma cells in the neuropil remote from blood vessels. In cases with severe encephalitis some of the perivascular cuffs are widened by loosely arranged macrophages and glial cells extending into the parenchyma. Focal gliosis may occur in rabies but is much less frequent than perivascular cuffing. In skunks inoculated intracerebrally with ERA^R rabies virus, there is a very severe encephalitis frequently with extensive regional accumulation of macrophages in the cerebellar molecular layer and in the cerebral cortex.

A degenerating neuron surrounded by macrophages and occasionally other inflammatory cells is referred to as a neuronophagic nodule or Babes' nodule. The central neuron is frequently shrunken and/or fragmented. Neuronophagia is infrequent in skunks.

A few degenerating neurons are usually present in rabies, but their numbers are much less than the number of antigen-containing neurons as detected by immunofluorescence. Except for the presence of antigen, most infected neurons are morphologically normal. In our experience neuronal degeneration is more common in the bovine and fox than in other species and the extent increases with increasing duration of clinical signs. Neuronal degeneration of ischemic type (shrunken

eosinophilic neurons) is more common in Purkinje cells than in other neurons. Neurons with complete or central chromatolysis occur infrequently. In a recent human case with a protracted period of clinical signs, there was marked loss of Purkinje cells and of neurons in the cerebral cortex (176). Similarly, marked neuronal degeneration was reported in rabid swine (177). As mentioned previously, neuronal degeneration is much more common in dorsal root ganglia than in the CNS and, in skunks, occurs in both immunocompetent and immunosuppressed animals (124).

The different mechanisms responsible for paralytic and furious rabies have not been clearly established. According to Chopra *et al.* (128), paralytic rabies in man is characterized by nerve cell destruction, microglial proliferation and perivascular infiltration mainly in the spinal cord and brain stem, whereas in classical (furious) rabies the inflammatory reaction, vascular changes and inclusion bodies are much more widespread and include the thalamus, hypothalamus, cerebellum and cerebral cortex (128). However, the lesions in paralytic rabies are not always confined to the brain stem and spinal cord (152). No reports comparing the distribution of antigen in the two forms of the disease have been found. Such examinations will likely be necessary to begin to understand the differing impairments of neural function in these differing forms of the disease.

Spongiform Lesions

The occurrence of spongiform lesions in the brains of rabid animals was reported in 1984 (178). This vacuolation of the neuropil was detected first in experimental rabies in skunks and foxes, and later in naturally occurring rabies in the following species: skunk, fox, horse, cow, cat, sheep. The lesion is considered to be "spongiform change" as defined by Masters and Richardson (179). Experimentally infected mice and rats did not have brain vacuolation. Qualitatively the lesions are identical to the vacuolar lesions of the transmissible subacute spongiform encephalopathies (SSE) although small vacuoles are generally less frequent in rabies than in the SSE, and progression to overt status spongiosis is less common. Light microscopically, the vacuoles are 2 to ~60 um and occur in the neuropil of the grey matter,

rarely in neuronal perikarya. The thalamus (all nuclei) and the cerebral cortex (inner layers) are the most frequently and severely affected areas. Other regions of the brain that are affected somewhat less frequently include the following: brain stem reticular formation, brain stem nuclei caudal to the diencephalon, cerebellar nuclei, cerebellar cortex and olfactory bulbs. Rarely other areas of the brain may be involved when the vacuolation is extremely severe in the above sites.

Studies to date indicate that rabies spongiform change develops very quickly (probably in less than 2-3 days), is not dependent on the immune response (occurs in immunosuppressed skunks), and occurs during infection with many different street virus variants (180). The development of the lesion is considered to progress through the following stages: formation of intracytoplasmic membrane-bound vacuoles in dendrites (less commonly axons and astrocytes), enlargement resulting in compression of adjacent neural tissue, rupture of the membrane-bound vacuole and surrounding plasma membrane, and herniation of adjacent cellular processes into the newly created tissue space. Generally the tissue space resulting from rupture of a membrane-bound vacuole is one of two types. In one, the boundary is smooth, fairly uniform and has continuous plasma membrane over large areas. In the other type (considered to be a more advanced lesion), the border is uneven being formed mainly by profiles of adjacent cellular processes protruding into the tissue space.

An important question is whether the membrane-bound vacuoles develop by incorporation of rabies viral antigen into the vacuolar membrane or whether the vacuoles develop from some indirect effect related to rabies viral infection. Two observations suggest that incorporation of viral antigen in vacuolar membranes is not required. First, budding virions have not been found on this membrane. Second, in extensive studies using the PAP technique, vacuoles occurred in several thalami containing very little antigen; some vacuolated areas were devoid of antigen. Also there was no correlation between amount of antigen and vacuolation in the cerebral cortex. Since most of the vacuoles begin in dendrites it is likely that the lesions are due to an indirect effect of rabies infection (180). Further studies of the

mechanisms involved should include determining the role, if any, of neurotransmitter imbalance (especially the excitotoxic neurotransmitters, glutamate and aspartate) in development of the vacuolation (180).

The reasons for lack of earlier reports (and presumed lack of identification) probably include reliance on the fluorescent antibody test for diagnosis during the past 20 years, use of small laboratory rodents in research, inadequate processing of tissues from rabid animals, and "conditioning" of pathologists to disregard "holes" in nervous tissue. For adequate detection and evaluation of the lesion by light microscopy, the brain should be removed soon after death, fixed in 10% neutral buffered formalin, dehydrated and embedded in paraffin. In our laboratory, use of a long (48 hr) processing cycle with chloroform as a clearing agent is effective in eliminating most artifacts that could be misinterpreted as spongiform lesions.

Most of the clinical signs of rabies are considered to be an expression of neural dysfunction. Recently, Gourmelon *et al.* (181) demonstrated changes in spontaneous electrical activity of brains in mice experimentally infected with field rabies virus. Three phases of the disease were recognized. They were as follows: initial phase (alterations of sleep stages, REM sleep disappearance, pseudoperiodic facial myoclonus and first clinical signs); mature phase (generalized EEG slowing); and terminal phase (extinction of hippocampal rhythmic slow activity). Brain electrical activity ceased 30 min before cardiac arrest. These studies, along with previous *in vitro* demonstrations of rabies-induced alteration of neuronal receptors for neurotransmitters, support the concept of impaired neuronal function in rabies (181).

PERIPHERAL DISSEMINATION OF VIRUS

During the spread of virus through the CNS, there is simultaneous centrifugal movement of infection in peripheral nerves (2,99). This accounts for the occurrence of virus in some tissues and fluids before the onset of clinical signs. The fact that saliva from clinically normal animals may be infective (for a few days before clinical disease) is well known and is the basis for public health measures dealing with biting animals.

Virions, matrix and anomalous viral products have been detected fairly regularly in axons of peripheral nerves (22,131) and only rarely in Schwann cells (131). Previous studies established that centripetal viral movement occurred via retrograde axoplasmic flow (112,113) and determined the rate of movement of virus (15). Tsiang (113) demonstrated centrifugal migration of virus in peripheral nerves after contralateral limb inoculation or inoculation of the CNS. The movement of antigen was blocked by application of colchicine to the nerve trunk (113). It is generally accepted that anterograde axoplasmic flow moves virus from neuronal perikarya in the CNS and cerebrospinal ganglia to peripheral tissues. By immunocytochemical methods, peripheral nerves contain linear arrays of finely granular antigen. It is likely that, with sufficient time, nearly all nerves of the body become affected. Although there is little direct evidence of specific types of fibers involved, the occurrence of antigen in intrafusal and extrafusal muscle fibers, epidermis, cornea and autonomic ganglia suggest that fibers of several types (myelinated, unmyelinated, motor, sensory, autonomic) may be involved.

Probably most of these viral products in peripheral axons are carried from neuronal perikarya by axoplasmic flow. However it has been suggested that some intra-axonal viral replication occurs and viral budding on smooth endoplasmic reticulum and the axolemma has been described in infected hamsters (131). This observation would seem to be inconsistent with the normal structure of peripheral nerve fibers and the mechanism of cell-to-cell transfer in the CNS. As mentioned above the peripheral processes of neuronal perikarya located in the spinal cord and cerebrospinal ganglia are morphologically axons and, thus, devoid of ribosomes that are required for viral protein translation. This problem, in fact, requires consideration in the mechanism(s) of release of virions from axon terminals. The mechanism of cell-to-cell transfer in the CNS involved budding on perikaryal and dendritic plasma membranes closely adjacent to sites of viral RNA replication and translation. Release from terminal axons would require some mechanism to circumvent the lack of ribosomes in axons and the long distances from perikarya to axon terminals. Possibly this could occur in one of several ways. Assuming replication in the neuron

of concern, these include: (i) transit of viral components (produced in the perikaryon) to the axon terminal, assembly of the various components into the complete virion (including budding on the axolemma); (ii) transport and release of virions synthesized in the perikaryon; and (iii) transport and release of subviral infectious particles. Alternatively, a mechanism of transcellular movement of virus without a replicative cycle (similar to that described by Tsiang (this volume)) could involve uptake by the cell body or central neurite, and axonal transport to the axon terminal where virus is released. (If such a mechanism occurs generally in rabies, it could account, to a large extent, for the very rapid dissemination of infection both in the CNS and via the peripheral nervous system to non-nervous tissue). Possibly, techniques for demonstration of viral RNA-specific proteins (182,183), *in situ* hybridization, and the use of nervous tissue explants (140) would contribute to such studies of the mechanism of transfer of infection from axon to non-nervous cell.

INFECTION OF NON-NERVOUS TISSUE

Many non-neural cells become infected as a result of centrifugal neural transport of virus. In various species, rabies antigen has been detected in cells of the epidermis, cornea, epithelium of the mouth, nasal mucosa and intestine, salivary glands, lacrimal glands, pancreas, intrafusal and extrafusal muscle fibers, myocardium, lungs, kidneys, adrenal medulla, and brown fat. The salivary glands are most important for spread of the disease and will be considered first.

Salivary Glands

For initial studies on isolation of virus from saliva see the section on entrance of virus into the animal body.

The amount of virus transmitted in natural contact between infected and non-infected animals in the wild is largely unknown. The results of experimental transmission in foxes suggest that the dose of virus (for fox-to-fox transmission) is in the order of 5,000 $MICLD_{50}$ (184, J. Blancou, personal communication).

There is substantial evidence that infection of salivary glands follows infection of the brain and that transport from the CNS is via

peripheral nerves (2,15,118,132,185,186). In skunks, widespread infection of salivary gland epithelial cells requires widespread release of virus from terminal axons (187). That is, cell-to-cell spread of virus among epithelial cells is not an important factor in development of extensive salivary gland infection.

Nearly all the extrinsic and intrinsic salivary glands can conceivably contribute virus to oral fluids. Most reports of salivary gland infection concern the submandibular salivary glands (SMSG). Naturally infected foxes, skunks, raccoons (*Procyon lotor*), cattle and deer have high rates of salivary gland (submandibular) infection (>80%) (188-190). Wandeler *et al.* (191) found that 93% of rabid foxes, 83% of rabid badgers (*Meles meles*) and 50% of rabid stone martens (*Martes foina*) had infected salivary glands. Apparently, insufficient numbers of naturally infected animals of other species have been examined to give meaningful data. Experimentally, high proportions of the following animals had virus in salivary glands: dogs, 61% and 74% (2,192); foxes, 100% (193); skunks, 76% (139); cattle, 80% (194); cats, 87% (195). The lacrimal glands of man (196) and cattle (197) may support replication of rabies virus. In experimental "derriengue", parotid glands contained virus at higher titer than SMSG (197). Virus was isolated from salivary glands of four of 14 experimentally infected hares (*Lepus capensis*) (198) and from none of 11 experimentally infected ferrets (*Mustela putorius furo*) (199).

Studies of skunks with naturally occurring rabies indicate that the following glands may contain antigen and/or infective virus: submandibular, parotid, sublingual, zygomatic, molar and lingual (188,200). Also the oral and nasal mucosa (mainly nasal glands) may support the growth of virus. Generally titers are high in the submandibular glands, moderate in the parotid, and low to moderate in the molar, sublingual and zygomatic glands. Occasionally the nasal glands may contain virus at higher titer than brain (188). We have not found reports of similar studies in other animals.

As mentioned above, the frequency of infection of SMSG is high in spontaneous rabies in several species, but there is little information on the frequency of low versus high titers of virus. Sikes (201) reported that salivary glands and saliva of foxes experimentally

infected with a fox (Alabama, U.S.A.) salivary gland suspension generally had lower titers of virus than glands and saliva from skunks similarly infected. In experimental studies using a skunk isolate (Wisconsin, U.S.A.) for challenge virus, Parker and Wilsnack (202) reported no difference in amounts of virus in salivary gland in the two species but did find greater quantities of virus in saliva of skunks than foxes. Foxes challenged with very high doses of challenge virus may contain virus at low titer in salivary glands (201). Experimental studies in dogs and cats (192,195) suggest that generally titers are slightly lower in SMSG of dogs than cats. Dogs inoculated with a Mexican isolate had SMSG with viral titers ranging from 10^1 to $10^{7.3}$ $MICLD_{50}$ /g (203). For the practical aspects of assessing the danger of exposure, any of the above species can have very high titers of virus in saliva and it would seem to be very difficult to determine the degree of risk by determining differences in viral titers in salivary glands.

In skunks (204) and dogs (J. Barrat and J. Blancou, personal communication) there is very little difference in viral titers or extent of immunofluorescence between right and left SMSG taken at the same stage of the disease (in skunks, <10 $MICLD_{50}$). This suggests generally synchronous infection and replication in the glands. Probably extensive interneuronal connections in the brain stem and spinal cord contribute to simultaneous, or almost simultaneous, infection of neurons that serve as pathways of infection to the salivary glands.

The marked variations in viral titer in SMSG that occur experimentally in skunks (204) and in other species (192,195,201) have not been fully explained. Possibly the experimental procedure including the type of challenge virus, dose, route of inoculation, and time of euthanasia influence the results (204).

Experimental studies in skunks suggested that the immune response could cause the following effects: (i) immune impedance of the process of infection of salivary gland epithelial cells and; (ii) neutralization of virus during the procedure of viral titration, thereby (in addition to (i)) depressing the amount of virus detected in salivary glands (124,204). Generally glands with small amounts of immunofluor-

escence had high tissue neutralizing antibody (TNA) titers and glands with low titers of TNA had extensive immunofluorescence (204). The minimal immunofluorescence was not considered to be due to blockage by antibodies (during the test) since other studies demonstrated that application of antibodies to rabies-infected tissue had little effect on the amount of immunofluorescence detected in standard tests (124). Thus the association of high TNA titer and minimal fluorescence in salivary glands is compatible with immune impedance of the process of infection of epithelial cells.

Although some depression of viral titer could be due to the above described impedance of the process of infection of epithelial cells, virus neutralization during viral titrations probably plays a significant role in production of low viral titers (second effect) (204). Salivary gland suspensions containing virus at high titer (and no antibodies) when mixed with skunk serum containing moderate to high levels of antibodies have a marked reduction in titer - even when all procedures are carried out at 4°C. The effect of antibody (in interstitial fluids) on virus contained in cells or in ducts before death is not clear. Lack of infectious virus in suspensions of salivary glands does not necessarily indicate that saliva excreted during or before the period of clinical signs was noninfectious (204).

Infectious virus may occur in saliva before the onset of clinical signs. The maximum reported preclinical periods are as follows: fox, 5 days (193); skunk, 6 days (204); dog, 7 days with a Mexican isolate and 13 days with an Ethiopian isolate (203); cat, 3 days (195); bat (*Tadarida braziliensis mexicana*), 12 days (85). Jonesco and Teodosio (205) demonstrated virus in SMSG of dogs 7 days before the onset of clinical signs. In experimentally infected skunks virus may occur intermittently in saliva, but in most cases virus is detected on or near the day of onset of clinical signs and continues until 1 or 2 days before death when saliva may again be negative. Vaughn (192,195) stated that there appeared to be a correlation between salivary gland and saliva titers in cats but not in dogs. Generally saliva viral titers were higher in cats than in dogs (192,195). Several claims of the carrier state in dogs indicate that, at least in some parts of Africa and Asia, excretion of virus can occur long before or even

without ensuing clinical signs (206-210). Studies with vampire bats (Desmodontidae) suggested that these animals could be carriers; i.e., infected bats could recover from clinical signs and subsequently excrete virus in saliva, or excrete virus without development of clinical signs (211). These conclusions (in bats) have not been supported by recent research (212).

Antigen, as detected by immunofluorescence and the peroxidase-antiperoxidase method, consists principally of small granules in acinar epithelial cells (156,187,213-216). Light microscopically, there may be accumulations of mononuclear cells in the interstitium and necrosis of scattered epithelial cells (1,215). Generally, necrosis of epithelial cells is more severe in the fox than in the skunk (215). In most skunks with rabies there is only slight accumulation of inflammatory cells and very few necrotic epithelial cells. Negri bodies may be detected in acinar epithelial cells of dogs (217); they are rare in skunks. Replication of rabies virus occurs in acinar epithelial cells (215,218) (mainly mucogenic cells in foxes) (218), with viral budding almost exclusively on plasma membrane (zones apical to nuclei). Release of virus particles occurs into intercellular canaliculi and acinar lumens. Virions in ducts are remarkably uniform and free from attached debris - suggesting a high infectivity: particle ratio (218). The preponderance of budding virions on plasma membrane (as opposed to intracytoplasmic membranes) is opposite to that found in neurons.

The role of auxillary factors (enzymes?) in saliva in augmenting the infectiousness of rabies virus was reviewed by Eichwald and Pitzschke (2). Some authors claimed an increase in pathogenicity due to saliva while others found no evidence to support this contention (2). This aspect of rabies infection would seem to deserve further detailed study in natural vectors of the disease using modern methods to characterize the viral strains.

Tsiang and Lagrange (219) suggested that injection of fox salivary gland homogenate could depress the cell mediated immune response in mice. It has not been determined if natural vectors of the disease would respond similarly or whether saliva (as opposed to salivary gland homogenate) would produce the same effect.

Non-nervous Tissues Other than the Salivary Glands

Concerning this aspect of the pathogenesis, very little useful new information has been published during the past 10-15 years. The following areas merit further study: the sites and mechanisms of adsorption of vaccine and street virus from the gastrointestinal tract (mainly to understand the immune response induced by orally-administered vaccines) (220); infection of the nasal mucosa (as a possible direct route to the brain even in animals immune to infection by other routes, and as a source of virus for oral fluids); excretion of virus in milk and urine (safety and public health considerations).

Rabies antigen has been detected in the following cells or tissues of the alimentary system: antigen in cells of the buccal and/or lingual mucosa of hamsters (58,131), mice (57,221) and skunks (188), and in the pancreatic acinar cells of hamsters (131) and foxes (214). In the respiratory system, the nasal mucosa of skunks (titer may exceed that in brain) (188), mice and hamsters (58,131) and bats (88), and bronchial mucosa of foxes (214) may contain antigen; virus has been isolated from lungs of hamsters (79,222), guinea pigs (83), and bats (85,88,223-227). In the urinary system, antigen has been described in the epithelial cells of the renal tubules, ureters, bladder, urethra and prostate of foxes (214), and the prostate of skunks (233); virus isolations were made from kidneys of infected bats (223), urine of naturally infected foxes (214,234,235), bats (236) and experimentally infected mice (58,237). Virus has been isolated from milk of rabbits (238), spotted skunks (*Spilogale* sp.) (239), guinea pigs, dogs, a woman (241), and from mammary gland tissue of bats (240) and sheep (242). Although transplacental transmission has been reported in man (243), cattle (244), mice (245) and skunks (246), it would seem to be uncommon (247). In the cardiovascular system, rare reports describe antigen in myocardial cells of hamsters (131); myocarditis (248,249), and Negri bodies in Schwann cells have been described in the human heart (250). In skeletal muscle of skunks, antigen (as a result of centrifugal viral migration) has been reported in scattered fibers in many muscles (intrafusal more frequent than extrafusal) both early and late in the disease (124). Hamster myocytes become infected as a result of centrifugal migration of virus (131).

Examination of skin with the fluorescent antibody technique can be used as an ante-mortem diagnostic technique in several species (251-259). It depends mainly on demonstration of antigen in nerve fibers surrounding hair follicles. Immunofluorescence has been described in cells of the epidermis of rabies-infected mice (mainly stratum granulosum and spinosum) (57) and skunks (257) and in the external root sheath of mice (57,58). Umoh and Blenden (259) mentioned antigen in cells of the stratum germinativum.

In the endocrine system, Negri bodies have been described in the adrenal medullary cells (1,217). Antigen occurs in the adrenal medulla of hamsters (131) and skunks (260), in the pineal gland of the skunk (260) and in the bovine hypophysis (261). Virus has been isolated from adrenal glands of foxes and skunks (at titers up to 10^4 $MICLD_{50}$/0.03 ml) (202). Accumulations of mononuclear cells occur frequently in the adrenal medulla of foxes and skunks (260) and have been described in man (250,262).

Rabies virus has been demonstrated in the brown fat of experimentally infected Mexican free-tailed bats (*Tadarida braziliensis mexicana*), little brown bats (*Myotis lucifugus*) and hamsters (263,264) and naturally infected insectivorous bats (223,236,265-268). Rabies viral strains differ in lipotropic characteristics (264,269,270) and strain modification can be induced by passage in cultures of brown fat (55). Bell and Moore (266) isolated rabies virus from the brown fat of *Myotis*. In a study of 1,717 bat submissions during a 5 yr period (including the species *Eptesicus fuscus*, *Myotis californicus*, *M. evotis*, *M. keenii*, *M. leibii*, *M. lucifugus*, *M. volans*, *M. yumanensis*, *Lasiurus borealis*, *L. cincereus*, *Lasionycteris noctivagans*, *Pipistrellus subflavus*, *Plecotus townsendii*) in Canada, Casey and coworkers did not isolate virus from brown fat of any bats that were rabies-negative by the fluorescent antibody test on brain (271).

The principal concern with brown fat is its role, if any, in long incubation periods or latency. Bats maintained in simulated hibernation retain viable virus (growing at a very low rate or not at all) for weeks or months and when returned to a warm environment, multiplication is initiated or increased (264,269,272). Rabies virus was maintained in cultures of brown fat kept at 8°C and, on transferring

cultures to 37.5°C viral multiplication increased (55,273). While these findings are not conclusive they suggest that brown fat can be a site of harborage of virus for later activation in induction of clinical disease.

In the special sense organs, Murphy *et al.* (131) described antigen in the taste buds of the tongue and virus has been isolated from various tissues of the eye (retina, choroid, vitreous body and lens) (232). In the cornea, infectious virus has been found predominantly, if not entirely, in the epithelial layer (90,274). Negri bodies occur in the cornea and in ganglion cells of the retina (2). Antigen was demonstrated in the cornea by immunofluorescence (237) and has been used in ante-mortem diagnosis (132). The recent transmissions of human rabies by corneal transplants indicate not only that the tissue contains infectious virus but that severed nerve fibers or reinnervation is sufficient for uptake and transmission of virus to the CNS.

VARIATIONS IN THE PATHOGENESIS

For a relatively coherent perspective of the disease "rabies" it is convenient to imagine the pathogenesis as being loosely categorized into two general types. In type 1, there is undelayed progression of the disease characterized by spread of the agent through the sites described above, development of the classical clinical signs, and death. Type 2 consists of variations from this standard that are due to facilitation of, recovery from, or delay in the progression of infection at one or more of the sites in the pathogenetic "flow" of infection. This type is considered to be relatively uncommon in the naturally occurring disease in a given species. Type 2 pathogenesis may be expressed in several different ways including recovery from infection (with or without clinical signs), recovery with chronic disability, shorter or longer incubation periods, prolonged or shortened periods of clinical signs, change in type of clinical signs, variations in excretion of virus, and the carrier state.

Although the precise viral and/or host factors responsible for these variations are largely unknown, the recent identification of street virus variants that are associated with species-specific

enzootic rabies (182,275-280), the "designing" of virus variants (281-288) (with markedly altered pathogenetic properties), and advances in nucleotide sequencing, immunology and immunocytochemistry are providing the tools to critically examine the relevant mechanisms. Such studies are important to understand the epizootiology of the disease, to develop control measures, and to determine the factors important in recovery from infection. For comprehensive discussions of genetically controlled resistance and the immune response, see the chapters by Drs. Lodmell and Macfarlan (this volume).

Evidence indicating recovery from or delay in the progression of infection at various sites is as follows: experimentally, virus can be prevented from leaving the inoculation site by denervation (48) or application of mitotic inhibitors (112,113) to nerve trunks. Possibly a similar mechanism (severance of nerves during biting) could occur naturally and account for the production of neutralizing antibodies in clinically normal animals (48,118,289-292). Baer and Cleary (18) found that in some experimentally infected mice (via the hind foot pad) infection did not progress beyond lumbar dorsal root ganglia (ganglioneuritis but no infection of the CNS).

Delay or impedance of centrifugal neural transport of virus would have little or no effect on the clinical outcome of CNS infection, but could reduce infection of the salivary glands and thereby reduce interanimal transmission. In some infected animals, virus may be absent from or present at low titer in salivary glands. Factors that affect salivary gland infection include the immune response, viral strain and rapidity of development of the disease (*vide supra*). Differences between street and fixed viruses for infection of salivary glands have never been adequately explained. This would seem to be a likely area for study of centrifugal axonal transport of virus and infection of nonneural cells.

The "carrier state" refers to chronic infection (with or without a period of clinical signs) with retention of the ability to transmit disease, usually by excretion of virus in saliva. Remlinger and Bailley (209) found a very low frequency of virus in saliva of healthy dogs. They considered that the infrequent occurrence did not warrant consideration in public health measures. Andral and Sêrie (206) isolated

rabies virus from saliva of clinically normal dogs or dogs with transient paralysis in Ethiopia. Fekadu and co-workers demonstrated that dogs infected with the "Ethiopian strain" could excrete virus in saliva for a long time without showing any signs of disease (208, 293,294). Experimentally, this strain appeared to produce the carrier state, whereas a rabies isolate from a Mexican dog did not (203). Similar findings have been reported from India (210). No convincing reports of the carrier state in naturally occurring rabies in North America were found.

Recovery from CNS infection can occur (albeit rarely) in both man and other animals (207,295-297). It appears that most of the rare cases of experimental or natural infections of birds are examples of recovery from infection either before or after virus reaches the CNS (102,298-300). Probably in most cases virus does not reach the CNS but induces an immune response as has been reported in birds feeding on prey or carrion (299). Reliable evidence of recovery from CNS infection includes isolation of virus or demonstration of antigen in tissues or fluids of animals (that subsequently recover) and neutralizing antibodies in CSF of recovered animals (301). Generally antigen and/or infectious virus are not detectable by standard methods after the lapse of a few weeks. That appreciable CSF antibody titers occur only with CNS infection is indicated by the general absence in hyperimmunized mice (301,302) and skunks (303). Antibodies in serum are not sufficient evidence of CNS infection since they may result solely from peripheral infection. Recovery from CNS infection may occur with or without sequellae. Apparently smoldering chronic CNS infection may be characterized by recurrent exacerbations (304).

For a given host species, several fixed or vaccine strains are avirulent or almost avirulent when given parenterally, but when given IC vary from being apathogenic to being rapidly lethal. Those apathogenic by the IC route frequently cause CNS infection which is overcome by host defences (immunosuppression usually converts a non-lethal infection into a lethal one). Flury HEP and other strains have been used to study the effects of various facets of the immune response in recovery from CNS infection (305-310). In addition to the fixed strains, street virus that is pathogenic for mammals will produce

abortive rabies in chickens (clinical signs may develop but later regress) (132). In most of the above systems, there is a period after inoculation during which virus replicates and antigen is detected in the CNS. This is followed by gradual loss of both infectious virus and antigen (as demonstrable by immunofluorescence).

The CVS strain of rabies virus is ordinarily pathogenic for mice. Modification of the glycoprotein of this strain through growth in the presence of selected antiglycoprotein monoclonal antibodies has produced variants, some of which are avirulent (by IC route in mice) (281-283). The avirulence is associated with changes in site III of the glycoprotein that are due to substitution of specific amino acids (286,287). In different studies, the apathogenic strains have been characterized by loss of the capacity to invade specific types of peripheral nerve fibers, reduced rate of spread in the brain (91,285), reduced rate of internalization in tissue culture (285) and inhibition of cell-to-cell spread by rabies virus-neutralizing antibody as compared to the parent virus (285). One strain (RV194-2), apathogenic for mice (IC route), was pathogenic when given to skunks IC but not IM. This indicates the critical importance of species and route of exposure in assessment of pathogenicity (275). Each of the above 2 mutants has a single amino acid substitution of the glycoprotein molecule at position 333. This would also seem to be the site of mutation of the Flury HEP and Kelev strains (285).

The studies of apathogenic strains of rabies virus indicate that these strains replicate in the CNS and that the immune response is crucial for resolution of the infection without development of clinical signs. In addition to the totally apathogenic strains, there are, as mentioned above, strains that frequently produce a sequence of CNS infection, clinical signs and recovery with chronic disability (207); i.e., infection that is more severe than that caused by the totally apathogenic strains but less severe than that caused by rabies street virus strains. It would seem that in regard to pathogenicity there is a spectrum of rabies viral strains varying from the completely apathogenic to the generally lethal CNS infections caused by most of the street virus isolates. There are only a few reports of pathogenic variation among street viral strains. The Ethiopian strain studied by

Fekadu (*vide supra*) and other strains (207) that frequently result in abortive infections are less virulent than most other field strains.

Of interest is the possibility that the species specificity of particular street virus variants is due to differences in pathogenetic features of particular antigenic types. The suitability (or unsuitability) of a virus variant for a given species must be manifest in simple, clinical expressions of the disease (susceptibility, incubation and morbidity periods, excretion of virus in saliva, frequency of aggressive behaviour). There is evidence that the pathogenicity of mid-Atlantic raccoon virus differs from Canadian Arctic virus in disease expression in skunks (276). In titration of each virus in skunks, there was a marked difference in average morbidity periods (raccoon strain, 50 hr; Canadian Arctic strain, 180 hr). This could in part account for the lack of enzootic skunk rabies in the mid-Atlantic and south-eastern areas of the U.S. and the lack of enzootic raccoon rabies in Canada. Variations in susceptibility (LD_{50}) of, and excretion of virus by, foxes, dogs and raccoon dogs (*Nyctereutes procyonoides*) when inoculated with the strains isolated from these species (311) support the general concept of species-specific enzootic rabies caused by "biotypes" of rabies virus (275,311,312).

In addition to the differences in pathogenicity of various strains, there is the possibility of *in vivo* viral mutation associated with production of DI particles (313). Interactions between viruses and DI particles may contribute to persistent infections (313). Blancou has suggested that modification of a strain (during the course of an epizootic) may halt or alter the spread of rabies in the species of concern (311).

The immune response can, under certain conditions, modulate expression of the disease or even prevent development of the clinical signs (as in pre-exposure vaccination of man and other animals and in post-exposure treatment of man). Possible results of immune-mediated alterations of the infectious process include the following (most of those listed in the first paragraph of this section): recovery from infection (with or without clinical signs), recovery with chronic disability, shorter or longer incubation and/or morbidity periods, change in type of clinical signs, and variations in excretion of virus.

The extent to which there is immune modulation of the naturally occurring disease is largely unknown. Several experimental studies have found that various aspects of the immune response were correlated with one or more of the above altered expressions of the disease, but there is very little information on specific immunologic reactions in the tissues of concern. The occurrence of rabies serum neutralizing antibodies in clinically normal wild animals (118,289-292) suggests recovery from infection (especially in those animals kept for prolonged periods after capture). In most cases of rabies in man, there is no evidence of immune responses until late in the disease (usually 7-10 days after onset of clinical signs) (122). Experimentally, skunks given high doses of street virus had serum neutralizing antibodies by 7-10 days after inoculation, but those given low doses had no detectable antibodies until clinical signs developed (204). Rabies occurred in both groups and only 1 skunk in the high dose group that developed serum neutralizing antibodies survived (204). Other features of the disease that are correlated in general with the immune response or with specific aspects of it include the following: increased mortality of mice (infected with street virus) due to immunosuppression (310); no increase in mortality of mice (infected with CVS or street virus) associated with immunosuppression (305,306); delay in onset of clinical signs of mice (infected with street rabies or Lagos bat virus) with immunosuppression (310,314); shortened incubation period of skunks (infected with street virus) due to immunosuppression (124); "early death" phenomenon in mice due to antibody and not to immune T-cells (315); optimum clearance of rabies virus (HEP) from the nervous system of infected mice requiring both B- and T-lymphocytes, although there is moderate reduction in mortality with either B- or T-cell response (306-309); immunosuppression-facilitated centrifugal neural migration of virus (2,310,316) and dissemination of virus to the salivary glands (204). Chronic rabies in experimentally-infected cats (304) was probably due to an immune response that delayed the outcome of the contest between virus and host.

In most of the disease models of recovery (mediated mainly by the immune response) from rabies there is infection of neurons in the CNS, albeit at a reduced rate. No reports were found to indicate whether

these infected neurons survive or are destroyed when the infection is eliminated from the brain. The availability of these disease models and the fact that, albeit rarely, some humans and other animals do recover from CNS rabies infection should be sufficient inducement for further studies of the mechanisms of recovery from this disease.

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6

GENETIC CONTROL OF RESISTANCE TO RABIES

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The studies of Webster and Clow with St. Louis encephalitis virus (1), and those of Lynch and Hughes (2) with yellow fever virus provided the first experimental evidence that the genetic constitution of the host can determine the outcome of mammalian viral infections. During these initial studies it soon became apparent that one could not speak of "virus-susceptible" and "virus-resistant" mice, but only of mice that were susceptible or resistant to a specific virus or group of viruses (3).

Since these early experiments, the majority of studies concerning genetically controlled resistance to virus infections in animals have been done in inbred strains of mice. Mice are the best species in which to delineate genetic determinants of resistance because there are a large number of inbred strains readily available, new congenic inbred strains can be easily developed, and an immense data base of information concerning murine genetics already exists (4). To date, murine genes that can specifically modulate the outcome of viral infections have been identified for 3 families of DNA viruses and 5 families of RNA viruses (5).

At the present time, our understanding of the nature of genetically controlled resistance to viral infections in murine systems can be generalized as follows (4): (i) a variety of genetic loci influence the outcome of infection; (ii) each locus affects responses to a single group of viruses; (iii) resistance may be either dominant or recessive; (iv) different susceptibility loci segregate independently and map to different regions of the genome; and (v) few resistance genes map to

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the H-2 region. (For more extensive reviews see Brinton and Nathanson (4), Brinton *et al.* (5), Bang (6), and Pincus and Snyder (7)).

At the time of this writing only 11 papers have been published concerning the genetic control of resistance to rabies. Four of these manuscripts were published from 1940-1964, and the remainder from 1979-1986. The paucity of research in this area more than likely occurred because few investigators believed that rabies virus-infected animals survived. Thus, resistance to rabies, let alone resistance that was controlled by host genes, was unimaginable. Today we know that these ideas were incorrect.

The first attempts to demonstrate that different strains of mice varied in their susceptibility to rabies virus were reported independently in 1940 by Johnson and Leach (8) and Habel (9). Although it is uncertain whether the mice used in these studies were inbred strains as we know them today, they had been continuously inbred for at least 4 years. In their studies, Johnson and Leach injected either of two different strains of rabies virus intracerebrally (i.c.) into mice from 10 different sources. It was determined that, with 1 exception, there was no noticeable difference in the susceptibility to rabies in the various strains. The exception was the Bar Harbor "Dilute Brown" strain which was slightly less susceptible to higher virus dilutions and rarely exhibited paralysis during the course of disease (8). Habel used a different approach in his studies in that he evaluated a rabies virus vaccine in different substrains of Swiss mice (9). It was determined that 2 of the 4 vaccinated substrains produced a higher degree of immunity upon i.c. challenge. This difference in resistance following vaccination was not attributed to the genetic constitution of the different substrains, however, but instead was attributed to the apparently older age of the resistant substrains. Additional studies to confirm whether age or genetic control accounted for these differences in immunity were not reported.

Twenty-two years following these initial studies, Dean and Sherman tested the potency of commercial rabies vaccines in different strains of mice (10). This study was followed 2 years later with an investigation concerning modified live rabies virus vaccines produced in embryonated chicken eggs and chicken embryo fibroblast tissue culture

(11). In the initial study, vaccines were tested with Albany standard, Albany Swiss, Swiss Webster and NIH standard Swiss mice. It was found that the vaccines offered greater protection in the Albany Swiss mice. Similar results were detected in the second study in that greater protection was obtained in Swiss than non-Swiss mice. Unfortunately, experiments were not done to determine why these mice varied in their susceptibility following immunization. Nonetheless, these vaccine studies did provide additional evidence that various strains of mice respond differently to rabies virus. More importantly, however, they also suggested for the first time that there might indeed be host genetic control to this virus.

Interest in genetic control of resistance to rabies did not resurface until 1979. At this time Nilsson *et al.* reported on rabies virus immunity in genetically selected high- and low-responder lines of mice that were produced by two-way selective breedings for maximal and minimal antibody production to flagellar (H/f and L/f lines) or somatic (H/s and L/s lines) antigens of salmonellae (12). The mice were immunized intraperitoneally (i.p.) with suckling mouse brain vaccine produced with challenge virus standard virus (CVS), and subsequently challenged i.c. Animals were bled before challenge to determine antibody titer. After specific immunization, both high-responder lines were more resistant to rabies virus infection than were the low-responder lines. Furthermore, resistance correlated directly with the level of serum neutralizing antibody in that antibody titers in both high lines were higher than the titers in low lines, although only the difference between H/f and L/f was highly significant. Thus, the difference in the resistance of the immunized high and low lines revealed an association with the nonspecific genetic control of antibody synthesis which the selective processes produced. Furthermore, these results also showed that nonspecific immune response genes may act on virus-specific immunity (12).

It is well known that the direct inoculation of rabies virus into the central nervous system (CNS) almost invariably results in death. Furthermore, it has been shown in many instances that the intramuscular (i.m.) or subcutaneous (s.c.) inoculation of rabies virus results in a high percentage of mortalities. Thus, if one had the inclination to

Table 1. Clinical Responses of Inbred Mice to i.p.-Inoculated SRV[a]

Mice	Clinical Response	Mortality
SJL/J and CBA/J	Resistant-Asymptomatic	0%
BALB/CByJ and DBA/2J	Resistant-Recovered	<10%
A/WySnJ and A.SW/SnJ	Susceptible	100%

[a] Mice were inoculated i.p. with 5 x 10^7 i.c. LD_{50} of SRV that had been isolated from an adult bat (*Eptesicus fuscus*) and then passaged 6 times by i.c.-inoculation in outbred Swiss-Webster mice.

study genetically controlled resistance to rabies, a route of infection other than i.c., i.m., or s.c. would have to be used. Ideally, this alternative route of virus inoculation would result in 100% mortality and 100% survival in different strains of mice. Furthermore, the route of inoculation should permit enough time for immunological and non-immunological (interferon, NK cells, virus attachment to and penetration of target cells) responses to the infection to occur before virus invades the CNS. It is for these reasons that I have chosen to do my genetic studies with i.p.-inoculated street rabies virus (SRV). To those who consistently criticize this route of inoculation, I realize it does not mimic a real life host-virus relationship or the natural route of SRV infection by bite. Nonetheless, this model system reveals clear-cut mouse strain differences in susceptibility, and it has permitted me to begin dissection of the mechanism(s) that are responsible for genetically controlled resistance to rabies.

In initial studies, 10 strains of inbred mice were inoculated i.p. with SRV (13). Three different clinical responses were defined among these mice: (i) SJL/J and CBA/J strains were invariably resistant and rarely developed clinical CNS disease (resistant-asymptomatic); (ii) BALB/cByJ and DBA/2J strains usually developed clinical CNS disease with irreversible paralysis, but most subsequently survived (resistant-recovered); whereas (iii) all A/WySnJ and A.Sw/SnJ mice died (susceptible) (Table 1). Interestingly, male mice of the BALB/cByJ and DBA/2J strains were less resistant than their female counterparts. C57Bl/10ScN, A/J, B10.A/SgSnJ and C57Bl/6 mice were of moderate and variable susceptibilities. Information on the comparative susceptibility of different strains of mice to experimental rabies also has been

presented by Blancou and associates (14). In their study, the susceptibility of 14 different strains or sub-strains of male or female mice were compared following inoculation of 1 - 2 LD_{50} of CVS into the masseter muscle. It was shown with mortality and incubation rates that the DBA/2 and nude strains were significantly less susceptible than the other strains. Interestingly, Blancou and associates have been unable to create a resistant strain of mouse by breeding survivors that had been inoculated i.m. or i.p. with CVS rabies virus (15).

Additional results from our initial studies showed that SJL/J mice were resistant to each of 6 different SRV isolates, whereas SRV resistant and susceptible strains of mice died following i.p.-inoculation with CVS rabies virus (13). Thus, a virulence factor(s) of CVS overcame the resistance mechanism(s) which prevailed against SRV. The resistant SJL/J and CBA/J strains also died after i.c.-inoculation with <=10 LD_{50} of SRV. It also was determined that i.c.-inoculated SRV replicated to equal titers in brains of resistant and susceptible strains of mice. Thus, genetic control of resistance was circumvented when SRV by-passed the immune system by being introduced directly into the CNS.

Challenge of F_1 hybrids produced by crossing resistant and susceptible strains of mice showed that resistance was dominant (97% survivors). Furthermore, resistance was not solely controlled by the major histocompatibility locus because susceptible A.SW/SnJ and resistant SJL/J mice have the same H-2^S haplotype. The number of genes controlling susceptibility was determined by inoculating backcross mice produced by mating F_1 hybrids with susceptible parents (13) (Table 2), or inoculating second backcross progeny produced from susceptible females mated with either randomly selected or rabies-resistant first-backcross males (16). The data strongly suggested that resistance was under the influence of either 1 or 2 genes. The reason for the slight variation in these results is unknown, but it appeared to be dependent on the strain and/or sex of the susceptible parent used to produce the backcross progeny. Furthermore, the =>96% resistance of offspring produced from (SJL X CBA)F_1 and (CBA X SJL)F_1 hybrids crossed to susceptible A.SW/J or A/WySn/J mice demonstrated that the resistance genes of SJL/J and CBA/J mice are allelic (16).

Table 2. Resistance to SRV of Backcross Mice Produced by Mating F_1 Hybrids with Susceptible Parents[a]

Backcross (female X male)	Survivors/total	
	Female	Male
(A/WySn X SJL)F_1 X A/WySn	74/128 (58%)[b]	68/121 (56%)[b]
A/WySn X (A/WySn X SJL)F_1	24/ 38 (63%)[c]	19/ 33 (58%)[c]
(SJL X A/WySn)F_1 X A/WySn	53/ 95 (56%)[b]	35/ 83 (42%)[b]
(SJL X A.SW/Sn)F_1 X A.SW/Sn	60/ 75 (80%)[d]	40/ 64 (63%)[c]

[a] 8-15 wk-old backcross mice were inoculated i.p. with 5×10^7 mouse i.c. LD_{50} of SRV. The experiments were terminated 21 days after inoculation. Control resistant SJL and susceptible A/WySn mice were included in all experiments.

[b] Results are statistically significant for 1 gene controlling susceptibility.

[c] Results are statistically significant for 1 or 2 genes controlling susceptibility.

[d] Results are statistically significant for 2 genes controlling susceptibility.

In a continuation of our studies, we examined 7 strains of mice to determine why susceptibility differences and variations in clinical CNS disease occurred among these animals (17). Experiments to determine spread and titers of infectious virus in various parts of the CNS indicated that these susceptibility differences were associated with restriction of virus replication within the CNS, and failure of virus to ascend the spinal cord to the brain. For example, virus replication in spinal cords of resistant-asymptomatic SJL/J and CBA/J mice was transient in that virus was not detected in this tissue after day 7 post-inoculation (p.i.). Furthermore, virus seldom was detected in brains of these mice. In contrast, virus replicated to high titers in spinal cord and then ascended to the brain in the resistant-recovered BALB/C and DBA/2J strains. At 21 days p.i. virus was not present in spinal cords or brains of these mice. High titers of virus were pres-

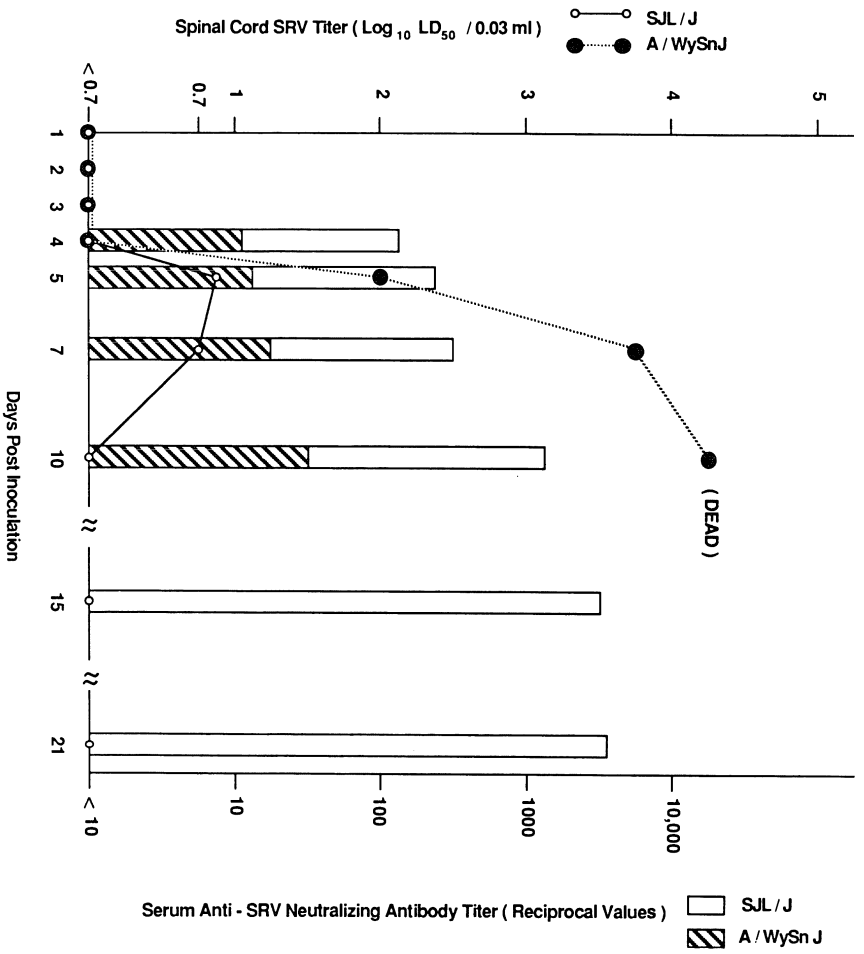

Figure 1. Comparison of spinal cord virus titers and serum neutralizing antibody titers of genetically resistant (SJL/J) and susceptible (A/WySn/J) mice. The 8-12 wk-old female mice were inoculated i.p. with 5×10^7 mouse i.c. LD_{50} of SRV. At least 6 mice of each strain were tested at each interval.

ent in spinal cords and brains of the susceptible A/WySn/J, A.Sw/SnJ and nude mice from the 5th day p.i. until their death. Restriction of viral replication appeared to correlate with the immune response in that prominent serum anti-SRV neutralizing antibody titers were detected in resistant strains, whereas susceptible strains produced only minimal amounts of antibody until their death (Fig. 1).

The importance of the immune response in this resistance was reaffirmed when it was determined that resistant SJL/J mice died after

immunosuppressive treatment with cyclophosphamide (17). Although the immunosuppressed SJL/J mice which were destined to die were not producing neutralizing antibody 7 days after virus infection, it was assumed that other immune factors such as cytotoxic T-cells also were affected by the cyclophosphamide treatment. Nonetheless, immunosuppressed SJL/J mice were protected by the passive transfer of immune serum up to 72 hr after i.p.-inoculation of SRV. Additional studies showed that there was no correlation between the appearance of antibody in the cerebrospinal fluid (CSF) and the resistance of the asymptomatic SJL/J and CBA/J mice. However, CSF antibody did appear to be associated with the survival of the resistant-recovered BALB/cByJ and DBA/2J mice that had developed clinical CNS disease (17). Our recent *in vitro* studies provide additional evidence that neutralizing antibody is important in resistance to rabies virus (18). It was determined that both antirabies virus immune sera and neutralizing anti-glycoprotein monoclonal antibodies inhibited the cell-to-cell spread of SRV, CVS and ERA rabies viruses in cultures of neuroblastoma cells and of non-neuronal baby hamster kidney (BHK-21) and chicken embryo related (CER) cells. Nonetheless, because antibody was more effective in inhibiting viral spread in the fibroblast and epithelioid cells than in the neuroblastoma cells, I suggest that the *in vivo* inhibition of viral cell-to-cell spread by antibody would more likely occur at an initial site of exposure and before nerves are infected (18).

Recent work by Templeton and coworkers also determined that susceptibility to murine rabies infection is genetically controlled (19). The protocol for their studies was different from ours, however, in that they used mice that had been immunized i.m. with a rhesus diploid cell line vaccine prior to s.c. CVS challenge in the ventral cervical region. Immunized mice were used because 100% of unimmunized mice died. In these studies C3H/J mice were determined to be hyper-responders (serum neutralizing antibody titer >50.63) and C57Bl/6J mice were hyporesponders (serum neutralizing antibody titer <=50.63). Furthermore, after vaccination, the C3H/J and (C3H/J X C57Bl/6J)F_1 hybrids were relatively resistant to challenge, whereas the C57Bl/6J were relatively susceptible, indicating that a dominant gene(s) controls survival. This resistance gene was not linked to H-2. Testing

Table 3. Analysis of Segregation and Linkage of H-2 Haplotypes, Serum Neutralizing Antibody (SNAb) Response to Rabies Vaccination, and Survival After Rabies Challenge Infection in BC[F_1(C3H/J X C57BL/6J) X C57BL/6J] Progeny[a]

H-2 Haplotype[b] and SNA[b] response[c]	Response to Challenge Infection				
	Survival		Death		
	No. of BC progeny	GMT[d]	No. of BC progeny	GMT	Days until death
b/k					
hyper	6	123	8	157	8-14
hypo	5	27	4	23	10
b/b					
hyper	1	149	4	148	10-12
hypo	2	23	7	25	10-12

[a] Mice were inoculated i.m. with a rabies vaccine derived from a rhesus diploid cell line. The CVS challenge of 3.5 x 10^5 tissue culture infective doses was given s.c. in the ventral cervical region. For more detail see ref. 19.

[b] H-2 haplotypes: C3H/J = H-2^k = k, and C57BL/6J = H-2^b = b.

[c] SNAb hyperresponders with individual SNAb titers of >50.63; SNAb hyporesponders with individual SNAb titers of <=50.63.

[d] GMT = Geometric mean serum neutralizing antibody titer.

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of backcross progeny produced between (C3H/J X C57Bl/6J)F_1 hybrids X hyporesponder C57Bl/6J mice showed that H-2, serum neutralizing antibody response to vaccination, and resistance after rabies infection segregated as monogenic, unlinked traits (Table 3). Thus, serum neutralizing antibody hyper- and hyporesponsiveness are controlled by single dominant and recessive alleles, respectively, and survival after rabies infection is dominant to nonsurvival (19). Interestingly, Templeton's evidence for the non-linked resistance gene supports previous suggestions (20-23) that serum neutralizing antibody alone does not provide complete protection to rabies infection. This

evidence is illustrated in Table 3 with backcross mice in that both hypo- and hyperresponders either survived or died following rabies challenge.

At this time I do not know the mechanism(s) of action of the rabies virus resistance gene(s). It is known, however, that resistance is dominant, controlled by 1 or 2 genes, and not linked to the H-2 locus or the serum neutralizing antibody response gene. Furthermore, it has been shown that rabies virus kills resistant and susceptible strains of mice following i.m., s.c., or i.c. inoculation. When rabies virus is inoculated i.p., however, the spread of infection within the CNS of resistant mice is self-limiting. These data suggest that the action of the gene(s) is probably at the cellular level. The expression of resistance at this level is most likely controlled through collaboration with the immune response because immunosuppression of resistant animals converts an asymptomatic infection into a lethal one. It also must be kept in mind that all survivors, whether immunized or not, produce neutralizing antibody.

To more completely understand the complexity of the mechanism(s) of murine resistance to rabies virus it will be necessary to evaluate the importance of systemic and CNS interferon during infection. In addition, the cytotoxic T-cell mediated immune response must be studied, as well as the attachment, penetration, replication and spread of virus in target cells at the initial site of CNS infection. Defective interfering (DI) particles also should be evaluated. The effect of DI particles on resistance could be especially significant if it is determined that they are more readily formed in resistant mice and subsequently interfere with standard virus replication. The feasibility of elucidating the mechanism(s) of resistance also will be enhanced by examining viral replication, as well as the effects of immunological and non-immunological factors on this replication, in tissue cultures prepared from CNS tissues of resistant and susceptible strains of mice.

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7

IMMUNE RESPONSES TO RABIES VIRUS: VACCINES AND NATURAL INFECTION

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INTRODUCTION

The first rabies vaccine was prepared by Pasteur over 100 years ago, and was used more or less successfully (1). Since then, rabies vaccines have been improved to the point where the recipient can be confident of surviving both the vaccination and exposure to rabies virus. This considerable achievement was made almost entirely by empirical means, that is by formulating various vaccines and testing their efficacy in appropriate animal models. It is therefore pertinent to ask the question as to what contribution modern immunology has to make to rabies prevention and prophylaxis. A partial answer is that a better understanding of the immune response to rabies virus and its antigenic components will allow us to formulate the next generation of rabies vaccines. Some improvements that could be made are:

(i) Economy. The current rabies vaccine recommended for use in humans is produced in human diploid cell culture and is therefore prohibitively expensive for use in most of the world. More relevant for veterinary medicine is the duration of immunity following vaccination. If this can be improved, significant reductions in the cost of rabies control programs will result.

(ii) Routes of administration. One of the aims of rabies control programs is to reduce the frequency of rabies virus in indigent species. In the case of wildlife, it is obviously not practical to vaccinate by conventional means; therefore, there has recently been a lot of interest in oral rabies vaccines which could be distributed in baits.

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(iii) Safety. It is generally considered that the fewer components in a vaccine the less the risk of unwanted side reactions. This is one of the strongest arguments for the introduction of subunit vaccines, especially for human use.

Several other papers in this volume deal with specific approaches to improving rabies vaccines to meet the above objectives. My aim is to focus on some of the basic immunological principles that are important either for protection against rabies infection or in the development of subunit and/or recombinant vaccines. I will summarize some recent results on the characterization of rabies virus antigens which I believe may be particularly relevant to vaccine development, and I will also make an attempt to evaluate which immune effector mechanisms are important in protection against rabies.

IMMUNOGENICITY OF RABIES VIRUS ANTIGENS

The rabies virion consists of 5 structural proteins, including a single transmembrane glycoprotein which is assembled as a trimeric spike (2). This glycoprotein is responsible for the initial binding interaction during the infection of susceptible cells (3), and is also the only target for virus neutralizing antibody (2,4). For this reason much attention has been focused on its possible use in a subunit vaccine. This field has recently been reviewed by Wunner *et al.* (5), and can be summarized by saying that, if adequately presented, the purified rabies virus glycoprotein protects experimental animals against rabies as effectively as vaccines consisting of inactivated virus. In a comparative study, Dietzschold *et al.* (6) compared the neutralizing antibody responses induced by monomeric "soluble" glycoprotein (a form of the glycoprotein secreted by infected cells, and lacking the hydrophobic transmembrane amino acid sequence), aggregated glycoprotein (rosettes), and glycoprotein inserted into lipid membranes (liposomes). Their conclusion was that, although each of these forms of glycoprotein carried all the antigenic information needed for induction of virus-neutralizing antibody responses, immunogenicity (as measured by induction of virus-neutralizing antibody and protection against challenge) was directly dependent on the state of

aggregation of these glycoprotein vaccines (6). It was subsequently shown that this finding also held true for cytotoxic T-cell (CTL) responses; that is, CTL could only be stimulated using the isolated glycoprotein if it was inoculated into mice in the form of liposomes (7). The natural conclusion from this work is that a subunit vaccine based on the rabies virus glycoprotein may require some type of lipid vehicle/carrier to be fully effective. Two particularly interesting methodologies for augmenting immunogenicity in this manner are the immunosomes described by Thibodeau *et al.* (8) and the structured complexes formed using the glycoside quil A (ISCOMS) (9). The evidence reported by these authors indicated that these structured complexes were considerably more immunogenic than simple liposomes (10).

The detailed antigenic structure of the rabies virus glycoprotein has been the subject of ongoing work. Using a panel of virus-neutralizing monoclonal antibodies, Lafon *et al.* were able to select neutralization resistant variants of both the CVS-11 and ERA strains of rabies, and to use the technique of cross-neutralization analysis to demonstrate at least 3 separate antigenic regions on the glycoprotein (11,12). This work has provided a framework for the analysis of the antigenicity of street rabies viruses using the same panel of monoclonal antibodies, and allowed the conclusion that street rabies viruses are heterogeneous with respect to these antigenic sites (13,14). Since a vital question for vaccine development is whether a single rabies vaccine can provide universal protection against all rabies strains, this result prompted cross-protection studies designed to address this point directly. Despite some quite significant antigenic differences between rabies viruses (as detected with these monoclonal antibodies), this did not result in vaccine "failure", although quantitative differences in the levels of cross-protection could be seen in certain combinations of vaccine and challenge virus (13,15). These conclusions, however, may depend somewhat on the experimental model used in the cross-protection studies (16).

In another approach for the immunological analysis of the rabies virus glycoprotein, peptides were prepared by treatment of the purified protein with cyanogen bromide and isolation of the cleavage fragments by electrophoresis under reducing conditions. Only 3 out of 7 of

these fragments induced significant virus-neutralizing antibody following inoculation into mice, and the levels of antibody induced were substantially less than obtained following inoculation with the intact glycoprotein (17). This finding was followed up by a series of experiments examining the immunogenicity of synthetic peptides based on the sequence of the rabies glycoprotein. Disappointingly, the levels of antibody produced indicated that a synthetic vaccine against rabies virus is an unlikely proposition. This is perhaps not surprising in view of evidence that even mild reduction abrogates the antigenic activity of the glycoprotein (5). One particularly interesting finding, which could have implications for the production of a subunit vaccine, arose from experiments designed to locate the intrachain disulfide linkages in the glycoprotein. Analysis of the cyanogen bromide fragments generated under non-reducing conditions showed that 1 particular peptide was entirely covalently linked by disulfide bridges to either of 2 other peptides (17). This evidence of 2 quite distinct patterns of disulfide bonding, and the fact that these 2 patterns were present in a 1 to 2 molar ratio, was interpreted to mean that the trimeric glycoprotein spike consisted of 2 forms of the monomer. These forms were considered to be identical in primary sequence but different in secondary structure. This unusual arrangement is presumably that which results in the lowest energy for the quaternary structure. In some strains of rabies virus (such as the CVS and PM strains of fixed virus) this difference in secondary structure results in the non-utilization of 1 of 2 glycosylation sites. This is the molecular explanation for the existence of the so-called G1 and G2 forms of glycoprotein differing in electrophoretic mobility (18). The importance of this finding to vaccine development is that this structural difference lies in the area characterized as being important for immunogenicity, at least on the basis of the peptide-immunization experiments (17). If these 2 forms of the glycoprotein differ in their immunogenicity, then it will be essential that glycoprotein produced for vaccine purposes by recombinant DNA methodology folds to give the optimum secondary structure.

Although analysis of the immunogenicity of peptides was not particularly profitable for studying antibody responses, this approach

led to the identification of at least 3 domains of the rabies virus glycoprotein which could restimulate proliferative responses by T-cells. In these experiments A/J mice were primed with inactivated rabies virus (strain ERA), and the proliferation of splenic T-cells was determined after exposure to appropriate rabies virus antigens or peptides. One of these domains was subsequently synthesized as 21 and 13 residue synthetic peptides; however, no evidence of protective activity was obtained (19).

It is generally considered that viral replication is required for the efficient generation of CTL responses, especially for viruses that do not fuse with host cell membranes at physiological pH (20,21). However, early work showed that this might not be the case for rabies virus. Wiktor *et al.* (22) found that inactivation of rabies virus by beta-propiolactone did not affect its capacity to stimulate CTL capable of lysing rabies virus-infected target cells. This unusual finding prompted experiments to determine the smallest viral component capable of stimulation. The experimental system used was similar to that described above, except that the assay was lysis of rabies-virus infected target cells as measured using a ^{51}Cr release assay, rather than proliferation. It was found that not only did purified glycoprotein stimulate a strong secondary CTL response *in vitro*, but that this response could actually be enhanced by fragmenting the glycoprotein using cyanogen bromide (7). The ability of the glycoprotein to serve as a stimulator of rabies virus-specific CTL was confirmed in 2 other ways. First of all, purified glycoprotein presented in lipid vesicles was able to stimulate a moderately strong CTL response following intraperitoneal inoculation of A/J mice (as noted above) (7). Secondly, infection of appropriate target cells with a vaccinia recombinant virus expressing the rabies virus glycoprotein rendered them susceptible to lysis by CTL generated following inoculation of mice with inactivated rabies virus (23). In the reverse experiment, infection of A/J mice with the vaccinia recombinant virus resulted in the generation of a population of CTL that was specific for rabies virus, as well as a population that was specific for vaccinia virus (23).

Given this information, the natural conclusion might be that the glycoprotein is the only rabies viral protein involved in protective

immunity. This conclusion may not be completely valid in view of evidence obtained from the analysis of rabies virus-specific CTL responses on a clonal basis (Macfarlan *et al.*, unpublished). A number of cloned CTL lines were developed from A/J mice inoculated with inactivated rabies virus (strain ERA), using methodology similar to that described by Braciale *et al.* (24). In order to ensure that the T-cell lines were (as much as possible) representative of the T-cell population, cloning was carried out at the first or second *in vitro* stimulation. As in other systems (24), all these CTL lines exhibited extremely high lytic activity. All were restricted by H-2K^k, and were broadly crossreactive amongst laboratory rabies virus strains (ERA, CVS-11, PM, Flury HEP and LEP) as well as some street rabies viruses that could be grown in the particular target cell line (the NA clone of the C1300 neuroblastoma cell line). Of special note was the finding that 3 out of the 8 CTL lines lysed target cells infected with the rabies-related Mokola virus. However, none of the cell lines lysed target cells infected with vaccinia-rabies glycoprotein recombinant virus, even though such target cells were susceptible to lysis by populations of CTL generated against inactivated rabies virions. The simplest interpretation of these findings is that the cloned CTL lines are specific for a viral protein other than the glycoprotein.

At the time that this work was done, the concept was somewhat heretical since the glycoprotein is the only viral component that might have been expected to be present on the surface of virus-infected cells. Similar findings have now been reported for a number of viral systems, however, and this type of result is no longer considered unusual (25). Further work on the specificity of these rabies virus-specific CTL clones awaits the development of recombinant viruses expressing other viral proteins; however, it should be noted that these results with cloned CTL lines are not quantitatively representative of those obtained using populations of CTL. In the latter case, there was no appreciable crossreactivity between Mokola virus and rabies viruses (14), and a sizeable proportion of CTL were in fact specific for the glycoprotein (23). This difference simply means that the selective pressures that operate *in vitro* in the cloning and maintenance of T-cell lines favor the emergence of a minority population of T-cells.

IMMUNE MECHANISMS IMPORTANT IN RESISTANCE TO RABIES

Several aspects of rabies pathogenesis are particularly important in considering immune mechanisms that might limit infection. First of all, there can be no argument that rabies virus spreads principally by cell-to-cell mechanisms. Moreover, the tropism of rabies is for sites that can be considered immunologically privileged by virtue of their location behind the blood/brain barrier (26). The cell-to-cell nature of rabies virus dissemination is demonstrated by experiments showing that the transmission of virus from a peripheral site of infection to the central nervous system (CNS) can be halted by neurectomy (27,28), and that in cell culture the spread of virus is not halted by an overlay of virus-neutralizing antibody (29). Another important factor in control of rabies infection is the stage of the disease. Following exposure to rabies virus, there is a short period of viability (as defined by the recovery of live virus from the site of trauma) during which there may be limited virus replication in myocytes near the site of infection (27). After this the virus disappears, and cannot be detected by any means until the next stage of the disease, which can be defined by the appearance of virus within the spinal ganglia or ventral motor neurons (27,30). Almost immediately after, virus can be detected within the brain (30). Since the spread of rabies virus within the CNS is extremely rapid, the chances of survival are much reduced if infection cannot be controlled prior to this stage. However, the existence of both experimental models (31,32) and clinical examples (33) of survival from rabies infection, accompanied by incontrovertible evidence of CNS involvement, indicates that this is not a universal rule.

From the above pathological considerations alone, it is possible to list the following "points of attack" for immune effector mechanisms to be useful in controlling rabies virus infection:

(i) Destruction of infected cells (relevant mechanisms include complement-mediated lysis, CTL, other cell-mediated effector mechanisms including those where specificity is provided by cytophilic antibody);

(ii) Prevention of the spread of virus to adjacent uninfected cells (mechanisms include neutralization of virus by antibody, sequestration of infectious immune complexes by phagocytic cells);

(iii) Prevention of infection of adjacent cells (by interferons, including gamma interferon produced by rabies virus-specific T-cells, and perhaps by other soluble mediators that alter the metabolic state of susceptible cells).

A major problem in making conclusions concerning the relative importance of individual immune effector mechanisms is the fact that the data on which such conclusions are based is often only applicable to a specific experimental situation. Having said this, at least in some experimental situations it has proved possible to protect animals against infection by passive transfer of antibody (34), suggesting that B-cells need to respond to rabies virus for successful immunization. Other evidence for the importance of neutralizing antibody responses comes from studies in which the correlation between protection from challenge and neutralizing antibody titer has been examined. In animals that have been vaccinated prior to challenge, this correlation is fairly good (35); however, no such correlation is seen if vaccination is delayed until or after the time of challenge (as has been done in order to provide an animal model of post-exposure vaccine treatment of humans) (36). Of interest is a report in which Miller *et al.* treated mice with anti-u chain antibody to produce B-cell deficiency (37). These animals displayed increased susceptibility to infection following intracerebral inoculation with the normally apathogenic Flury HEP strain of rabies virus, but were not as susceptible as completely immunosuppressed mice or mice that had been adult-thymectomized, irradiated, and reconstituted with bone marrow (T-cell deficient mice). This was interpreted to mean that both T- and B-cells were important for resistance to rabies virus. An important question raised by these studies is how antibody might affect the course of infection, particularly since in this experimental model virus is inoculated directly into the brain. If virus neutralization plays a role, then the virus must be accessible to virus neutralizing antibody. Iwasaki and Clarke have presented evidence that rabies virus spreads within the brain following release of infectious virus into cerebrospinal fluid-filled intercellular spaces, as well as by direct cell-to-cell mechanisms (38). Note that the distinction between these 2 modes of infection is likely to be very important, since it has been shown that the presence

of antibody has no effect on the cell-to-cell spread of certain rabies viruses *in vitro* (29).

Alternatively, the role of neutralizing antibody may be to prevent a second cycle of infection subsequent to destruction of infected cells by any of a number of cytolytic mechanisms, including complement-mediated lysis of antibody-sensitized cells (37). Bell *et al.* have provided evidence that the presence of virus-neutralizing antibody in the cerebrospinal fluid has a special significance for the survival of infected animals (39). It is important, however, to distinguish between antibody produced within the brain in response to local antigenic stimulus, and that detected in the brain late in infection after the blood/brain barrier has been breached.

It is clear that one vital function of T-cells is to provide help for the induction of T-dependent antibody responses to rabies virus. This is shown by the susceptibility of genetically athymic mice (nu/nu) to rabies virus-infection and their lack of neutralizing antibody responses (40). The more difficult question is whether T-cells might contribute directly to protection. There seem to be 2 possible mechanisms by which this could occur: (i) cytotoxic T-cells specifically killing rabies virus-infected cells, and thereby preventing spread of infection; and (ii) release of soluble mediators (such as interferon gamma) by specifically stimulated T-cells.

The first description of the induction of CTL responses by rabies virus was that of Wiktor *et al.* (41). They showed that mice exposed to live or inactivated rabies virus generated a strong CTL response which was specific for rabies virus-infected target cells, maximal at 6 days after infection, and required at least partial identity between the H-2 genes of the CTL and virus-infected target cell. The failure of passively transferred antibody to protect against rabies in a mouse model of post-exposure vaccine treatment led to the hypothesis that CTL might be important, since such mice were protected following vaccination regimes that induced CTL (36). This hypothesis gained apparent support from experiments dealing with the induction of CTL following intracerebral inoculation of mice with virulent or attenuated rabies viruses. It was found that survival from rabies directly correlated with the induction of CTL responses, and not with other immunological

parameters examined (antibody and interferon responses). In other words, attenuated viruses induced CTL responses whereas virulent viruses did not (36,42). This phenomenon was due to an active suppression of CTL responses, and not simply the failure of some rabies viruses to stimulate CTL, since mice infected with virulent rabies virus by the intracerebral route failed to generate CTL in response to a concurrent infection with influenza virus or to reject fully allogeneic skin grafts (42). To take these findings 1 step further, the T-cell subpopulations of lethally infected mice were directly enumerated using monoclonal antibodies specific for Thy 1, Lyt 1, Lyt 2, and Ia antigens. It was found that there was a virtual disappearance of the Lyt 2-positive lymphocyte subpopulation responsible for cytotoxic function (43). Since lethal rabies virus infection is also associated with a number of other effects on the immune system, including dramatic decreases in the weight and cellularity of secondary lymphoid organs as well as thymic involution, it could be argued that the lack of CTL function simply reflects these stress-related effects. This was not the case since adrenalectomy reversed the loss of spleen weight and cellularity, but not the generation of CTL responses, of lethally infected mice (43).

The biological importance of this immunosuppressive mechanism remains to be seen, since all experiments have so far been carried out using a highly artificial model system. Two of the most important questions that remain to be answered are whether a similar phenomenon occurs in humans or other target species, and how this loss of CTL function affects survival from rabies.

The second mechanism whereby T-cells might play a direct role in controlling rabies virus-infection is by releasing gamma interferon. This lymphokine can be produced by both helper/inducer (44) and cytotoxic (45) T-cells following antigenic stimulation, and is unrelated to other classes of interferons, except that by definition it confers resistance to viral infection on suitable target cells. Clearly this antiviral activity is 1 mechanism that could be beneficial in rabies virus infection. While not relating to gamma interferon, there is certainly experimental evidence that interferon inducers (36,46,47), or interferon preparations (47) can modify the course of infection in

various experimental models. Another effect of gamma interferon is to induce the expression of class 2 histocompatibility antigens on appropriate target cells (48). Since helper/inducer T-cells recognize antigen as a complex with class 2 histocompatibility antigens, this has a number of ramifications for the immune response induced by rabies virus and perhaps some aspects of immunopathology. However, experimental evidence is required before this can be evaluated properly.

One of the more surprising aspects of the immunopathology of rabies is the almost complete lack of an inflammatory response within the CNS (at least in non-immune animals). Generally the only histological findings are a mild perivascular infiltration of mononuclear cells in the brain stem, spinal cord, and ganglia (49,50,51), although exceptions have been noted (51). This contrasts with other viral diseases of the CNS where inflammation can be the major feature of the pathological picture (52,53). It is therefore necessary to ask how cell-mediated effector mechanisms can be postulated to play a role in resistance to rabies virus infection in the physical absence of a cellular response within the CNS. First of all, these findings do not relate to vaccinated animals since a successfully vaccinated animal will not be infected within the CNS. Secondly, a massive infiltration of cells need not occur for a fully effective local immune response, as demonstrated by the results of Ceredig *et al.* (53) who showed that very few T-cells infiltrating the CNS of lymphocytic choriomeningitis virus-infected mice were specific for the virus, although a substantial inflammatory response was in fact observed in this model and was presumably induced by these few antigen-specific T-cells. In addition, it may not necessarily be beneficial for there to be a gross inflammatory response within the CNS. One important consequence of immune effector mechanisms operating within the CNS is the potential for immunopathological damage. Smith presented definitive evidence that this can occur during rabies infection. She showed that cyclophosphamide-induced immunosuppression after street virus infection resulted in prolonged survival of mice, but that treatment of such mice with rabies virus-specific antibody induced paralysis followed by death (32). Paralysis was also associated with the return to immunoresponsiveness of virus-infected mice following cessation of the immunosuppressive regime. Thus it

appears possible that death from rabies is at least partly due to host immune mechanisms. It has also been shown that the phenomenon of "early death" following vaccination results from immunopathological mechanisms, although the validity of the experimental model remains questionable (54).

CONCLUSIONS

It is clear that many questions remain to be answered concerning the role of immunological mechanisms in protection against rabies. Perhaps the most important of these, from a practical point of view, is whether it is necessary to consider the capacity of a vaccine to stimulate T-cells in order to develop the next generation of veterinary and human rabies vaccines. The excellent correlation between antibody titers and protection seen in defined experimental models of pre-exposure vaccination indicates that this may not be necessary. However, this correlation has not been proven to have anything to do with the actual mechanism of protection. Although not a question which is of primary importance to veterinary medicine, the protective mechanism following post-exposure treatment of humans with rabies vaccine is very likely to involve T-cells. In any case, the progress made in applying new technology to the old problem of a better rabies vaccine indicates that such a vaccine will be developed. Hopefully progress will also be made in determining how rabies vaccines work.

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8

RABIES-RELATED VIRUSES

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ABSTRACT

Since, unlike most strains of classical rabies virus, the rabies-related viruses have been isolated from species other than carnivores, an attempt has been made to bring to the fore their virus-host relationships. Duvenhage virus, the only rabies-related virus to date found outside of Africa, now appears to be endemic among European bats, and the development of this epizootic is chronicled. Of considerable importance to public health authorities is the question of whether or not current vaccines protect against the European variants of this virus, and this topic is discussed in detail.

INTRODUCTION

Rabies in terrestrial animals has long been recognized as a terrifying disease. Pasteur, on whose classical experiments all subsequent work on rabies is founded, recognized that there were biological differences between the strains which cause the disease. By repeated passage of wild ("street") isolates intracerebrally in laboratory animals, he was able to alter their biological properties so that they became more neurotropic and the incubation periods became less variable ("fixed"). It was not until many years later, however, long after rabies had been shown to be caused by a virus, that antigenic differences between strains were recognized and their possible implications for vaccine programs were first considered. Within the past decade, the application of monoclonal antibody techniques has permitted the differentiation between viruses from different species

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and from different geographical locations. Nevertheless, such studies have led to the conclusion that, despite the variations, most of the strains in terrestrial animals can be regarded as classical (serotype 1) viruses.

Among the mammals, bat species are in number second only to the rodents, yet comparatively little is known of their natural history. It was from bats that the first truly distinctive rabies-related virus isolate (Lagos bat virus, serotype 2) was made, and this was followed by the isolation of another rabies-related virus from a man bitten by a bat (Duvenhage virus, serotype 4). Many further isolations of this virus from bats have since been reported in Europe.

Shrews are found on all major land areas, and the *Crocidura* species from which another rabies-related virus (Mokola, serotype 3) was isolated are common in Africa and Europe.

Two other viruses, Obodhiang and kotonkan, have been isolated from arthropods. They have been shown to be distantly related to each other and to Mokola virus.

Rabies is still one of the most important epizootic diseases of the world. The rabies-related viruses, though of less public health importance, may yet provide the information needed for a fuller understanding of the complex virus-host relationships of the disease.

THE RABIES SEROGROUP

Classical rabies, Lagos bat, Mokola, Duvenhage, Obodhiang and kotonkan viruses (Table 1) together form the genus *Lyssavirus* within the family Rhabdoviridae. All have a characteristic bullet shape (see Tordo and Poch, this volume). Since the relationship of the arthropod-borne members to the others is distant and only through Mokola virus, Bauer and Murphy (1) have suggested the need for further physico-chemical characterization to determine if they should be placed in a separate subgroup. However, in a recent review, Shope and Tesh (2) consider that these viruses should be included in two separate serotypes within the lyssaviruses.

Serotype 1: Rabies Virus

The prototype strain of the serotype is Challenge Virus Standard CVS-24, which was derived from Pasteur's virus. This serotype also

Table 1. Members of the Genus *Lyssavirus**

Serotype	Known geographic distribution	Source(s) of virus in nature
Rabies	Worldwide except Australia, New Zealand, Japan, United Kingdom, Antarctica, parts of Scandinavia, Hawaii, and some other islands.	Dogs, cats, wild carnivores, bats, cattle, humans
Lagos bat	Nigeria, Central African Republic, Republic of South Africa, Zimbabwe, Senegal	Fruit bats, cats
Mokola	Nigeria, Cameroon, Zimbabwe, Central African Republic	Shrews, humans, cats, dog, rodent
Duvenhage	South Africa, Zimbabwe, Europe	Humans, insectivorous bats
Obodhiang	Sudan	*Mansonia* mosquitoes
kotonkan	Nigeria	*Culicoides* midges

* After Shope and Tesh (2), with permission.

includes the majority of street viruses, whether isolated from dogs or cats among domestic animals, or from wild animals such as foxes, skunks and raccoons. Viruses isolated from bats in the Americas are included in this serotype (see Smith and Baer, this volume), as are the viruses from historical disease syndromes such as "oulo fato" (3), Nigerian Horse disease (Staggers) (4), and Derriengue (5) in vampire bats.

Serotype 2: Lagos Bat Virus

The knowledge that rabies virus may be carried by insectivorous and non-sanguinivorous bats suggested to Boulger and Porterfield (6) that an examination of frugivorous bats on Lagos Island in Nigeria might be of interest. They isolated a virus from the pooled brains of 6 *Eidolon helvum* bats, which are gregarious fruit-eaters, usually roosting in colonies of 20 to several hundred and sometimes as many as several thousand. The virus was pathogenic for 21-28 day-old mice, and though examination of infected mouse brains revealed cuffing of blood vessels and neuronal degradation, no Negri bodies were seen. Additionally, since the virus was not neutralized by a potent rabies immune

serum prepared in rabbits, they concluded that it was not a rabies virus. In 1970, however, Shope and coworkers (7) demonstrated that the virus was related to rabies virus, and that the degree of cross-reactivity among rabies, Lagos bat and Mokola viruses was sufficient to substantiate a distinctive serogrouping within the Rhabdoviridae. It was in their paper that the term "rabies-related viruses" was first used; it has remained in common usage ever since. In 1974, a second isolation of Lagos bat virus was made from a *Micropterus pusilus* bat in the Central African Republic (8,9). These dwarf epauletted bats are also fruit-eating, but they normally roost singly or in small groups and are seldom if ever found in association with other bat species.

In 1980, several isolations were made of Lagos bat virus from *Epomorphorus wahlburgi* bats from around Durban, Natal (10,11). These epauletted fruit bats roost in groups of up to 50, are common garden residents in the subtropical towns of Natal, and make local migrations in search of ripe fruit. According to the authors, there is overlap between the migratory ranges of *Eidolon* and *Epomorphorus* bats.

Two further isolates (SA2 and SA3) from unidentified South African bats, originally reported to be Mokola virus (12), were later shown to be Lagos bat virus (J.S. Smith, personal communication). Lagos bat virus was also identified in our laboratory from the brain of a domestic cat which died in the Republic of South Africa (A. King, unpublished data), and from a cat in Zimbabwe (C.M. Foggin, personal communication).

Serotype 3: Mokola Virus

During the course of a surveillance program designed to detect viral infections in the wildlife of Nigeria in 1968, virus was isolated from 3 shrews (*Crocidura* sp.) captured in the district of Mokola in Ibadan. The isolates, of which Ib An 27377 was selected by Shope and coworkers (7) as the reference strain, were grown in mice from shrew lung, liver, spleen and heart tissue pools, and intracerebral passage in 3-day-old mice resulted in death in 4 days. Within a short time, 3 further isolations of Mokola virus were made in Nigeria: from a liver and spleen pool from a shrew found dead in laboratory grounds (13); from the cerebrospinal fluid of a 3½ year-old girl with "aseptic meningitis" (14), and from a 6 year-old girl with symmetrical paralysis

observed rabies in 3 *Nyctalus noctula* bats in Yugoslavia (Table 2). These bats are insectivorous as are all European bat species. Nikolitsch (23) reported a further case in the same species in Yugoslavia in 1957. Tunçman (24) reported rabies in a *Rhinolophus ferrum-equinum* (horseshoe) bat in 1956 in Turkey. Whether or not the viruses which caused death in these bats were of serotype 1 or 4 is unknown since no material from the bats is available for analysis, and there have been no further reports of infected bats from either country.

In an enlightened observation, Mohr (25) drew attention to the fact that even in latitudes as far north as Hamburg, bats may be infected with rabies. His subject was the isolation in 1954 of rabies virus from a bat in Hamburg which died the day after it bit a boy on the finger as he removed the bat from a tree. No Negri bodies were seen in the bat brain, but large numbers were seen in mouse brains at the second passage. Subsequent events suggest that the bat infection was caused by a serotype 4 Duvenhage virus, but no material remains for analysis.

Wersching and Schneider in 1969 (26) reported a second isolation from a bat in the same city 14 years later in which "the biological properties of the virus isolated were found in part to differ considerably from known street virus infections". In neither incident was the species of bat identified, nor was that of a third infected bat found in 1970 at Stade, about 35 km west of Hamburg. Although in 1975, Hentschke (27) reported rabies in a *Myotis myotis* bat in Berlin, bat rabies was not diagnosed again in the Federal Republic of Germany until 1982, when an unidentified infected bat was found in Bremerhaven, nearly 100 km west of Hamburg. A further case was diagnosed in an *Eptesicus serotinus* bat in 1983, and by the end of 1985 it was clear that the disease had established itself in serotine bats in the Federal Republic of Germany, and it has continued to be reported. Characterization of the Stade virus with monoclonal antibodies (Mabs) identified it as a serotype 4 Duvenhage (28). In the meantime, Pitzschke (29) isolated "rabies" virus from a serotine bat in the German Democratic Republic.

Five incidents of bat rabies have been recorded in the U.S.S.R., 2 of which led to human fatalities. In 1964, Selimov and coworkers (28,

Table 2. Rabies in Bats in Europe: Primary Isolations

	Year confirmed	Year reported	First author	Text ref.
Yugoslavia[a]	1954	1956	Nicolic	22
Turkey[a]	1956	1958	Tunçman	24
Federal Republic of Germany	1954	1957	Mohr	25
German Democratic Republic	1963	1965	Pitzschke	29
U.S.S.R.	1964	1986	Selimov	30
Poland	1972	1974	Komorowski	31
Denmark	1985	1985	Bitsch	34
Finland[a]	1985	1986	Lumio	33
Netherlands	1987	1987	Neuwenhuis	-[b]
Spain	1987	1987	Mueller	-[b]

[a] No further isolations to date.

[b] Personal communication.

30) reported the isolation of rabies virus from a bat which died 25 days after biting a man in Kiev who was given vaccine and survived. In 1977, a young woman of Voroshilovograd died some 35 days after she was bitten on the forefinger by a bat, and in 1985 a child (Yuli) died 27 days after she was bitten on the face by a bat in Belgorod. Rabies was also confirmed in 1985 in a bat from Omsk which died 5 months after capture, and in a bat caught in Novosibirsk (28). It was concluded from Mabs studies that the Yuli virus isolate was a serotype 4 virus (28).

In 1974, Komorowski and coworkers (31) reported the first incident of rabies in a serotine bat from the region of Krakow in Poland. A second case was recorded in 1985 (32) when examination of a bat which had bitten a 3 year-old child in Gdansk, the Baltic Sea port nearly 500 km north of Krakow, revealed a serotype 4 virus.

Rabies was confirmed in a man in Helsinki, Finland, in 1985 (33). He had been bitten by a bat in Malaysia 4½ years earlier, by other bats in Switzerland 1 year and in Finland 51 days before his death. In preliminary studies with Mabs, the virus isolated was shown to be related to the rabies viruses isolated earlier from bats in Germany.

Towards the end of 1985, a serotine bat which had bitten a woman in Jutland was found to be infected with a virus belonging to the rabies group but not to serotype 1 (34). In that year, 9 further serotine bats were found to be infected, and 1986/87 has shown the disease to be endemic in Denmark (serotype 4).

Rabies in bats in the Netherlands was observed for the first time in May 1987 and by the end of September the disease had been confirmed in 78 serotine and 3 *Myotis dasycneme* bats (H.U.R. Neuwenhuis, personal communication). Also in 1987, bat rabies was confirmed on 2 occasions in Spain, in Grenada and in Valencia (W.W. Mueller, personal communication): 1 of these isolates was a serotype 4 virus (J.S. Smith, personal communication).

Other Rabies-related Viruses: Obodhiang and Kotonkan

Schmidt (35) proposed the name Obodhiang for 1 of 4 immunologically distinct viruses isolated in 1965 in infant mice from man-biting *Mansonia uniformis* mosquitoes collected at Malakal, Sudan. In Ibadan, Nigeria, Kemp and coworkers (36) in 1967 isolated from a pool of about 250 *Culicoides* midges a rhabdovirus (Ib AR 23380) which they named kotonkan, the Yoruba word for small biting gnats and meaning "almost nothing". Obodhiang virus was isolated on 3 separate occasions from unengorged *Mansonia uniformis* mosquitoes. The virus has not been associated with clinical disease in nature but newborn mice inoculated intracerebrally died 4-14 days later (1).

Kotonkan virus has been isolated once only, but it may be the cause of a disease in cattle that resembles bovine ephemeral fever (36). A mild clinical illness was induced in a susceptible calf (37) and neutralizing activity for the virus has been found in Nigeria in a high percentage of the sera of cattle, and also in sera of man, rats, sheep, horses, rodents and hedgehogs (36). Both Obodhiang and kotonkan viruses replicate in mosquito cells (38) and in mosquitoes, in which they pass into the salivary glands (39).

The relationship of these 2 viruses with Mokola virus, shown initially by Kemp and coworkers (36) using complement fixation tests, was confirmed by Bauer and Murphy (1). The 2 arthropod-borne viruses were passaged intracerebrally several times in infant mouse brains

before adaptation to vertebrate cell cultures, and the uncloned stocks were then used to infect suckling mice from which brain impressions were made. Indirect fluorescent antibody tests using dilutions of immune ascitic fluid revealed low levels of cross-reactivity between Obodhiang, kotonkan and Mokola viruses, but no reaction with Lagos bat and two classical rabies viruses used as controls. No cross-reactivity was found by neutralization tests or surface immunofluorescence.

Working with cloned material, however, Buckley and Tignor showed some degree of relatedness between the same 3 viruses by cross-plaque neutralization reduction tests in Vero cells (38). Mokola antiserum reduced Obodhiang and kotonkan plaque formation by 68% and 39% respectively, but the cross-reactivity was one-way since neither Obodhiang nor kotonkan antiserum reduced Mokola virus plaque formation (40).

The viruses are of interest since to date no other relationship has been established between rabies and insect-borne viruses. Mokola virus multiplies in both vertebrate and invertebrate cell cultures (40) and experimentally in mosquitoes (39), indicating that this virus may be a biological and serological bridging virus between rabies and the viruses Obodhiang and kotonkan (40).

STRUCTURAL RELATIONSHIPS

Rabies, Lagos bat, Mokola, and Duvenhage viruses are morphologically and morphogenetically very similar (41), all usually having uniformly cylindrical forms and a honeycomb structure which can be seen easily by electron microscopy (Fig. 1). Obodhiang and kotonkan viruses are characterized by cone-shaped particles (1), another feature which has also led to speculation that they may be more suitably grouped with bovine ephemeral fever virus, which sometimes has cone-shaped particles (42). However, electron microscopic observations may not be of too much significance when relating these viruses within the Rhabdoviridae, since some rhabdoviruses of fish are also characterized by the honeycomb surface structure (43) and the completely unrelated Marco virus also has cone-shaped particles (44,47). In our own hands Flury LEP virus cultured in BHK-21 cells frequently produces cone-shaped particles (unpublished data).

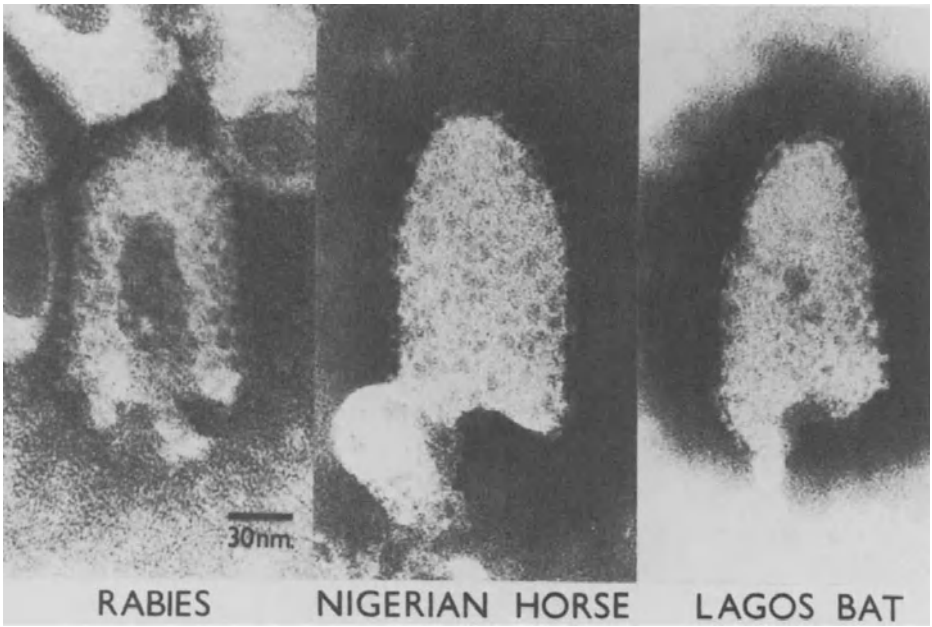

Figure 1. Electron micrographs of 3 lyssaviruses grown in BHK-21 cells and purified by sucrose density gradient ultracentrifugation. The classical rabies strain (at left) is Flury LEP.

One of the characteristics of the rhabdoviruses is their ability to produce defective interfering (DI) particles which, while antigenically similar to the parent, contain only part of the genomic RNA (45). It has been postulated that DI particles may have a role in the outcome of the infection *in vivo* (46) but there is still no clear evidence on this point (47,48). However, the ability of DI particles to interfere with virus production *in vitro* is regarded as specific for that particular virus. DI particles produced by a classical rabies virus (Flury LEP) interfered with the production not only of the homologous virus but also with that of Nigerian Horse (Staggers) virus and Lagos bat virus, suggesting a degree of genetic homology between them (J. Crick and F. Brown, unpublished data).

At the genomic level, little is known of the interrelationships between serotypes 1-4. The limited amount of information available concerning genome homologies between the viruses was cited in 1979 (49), but since then the techniques of hybridization on which these

studies were based have been largely superseded by cloning and sequencing studies. The complete genome of at least 1 of the classical rabies strains has been sequenced (50; see also Tordo and Poch, this volume), and the primary structure of the glycoprotein of another fully established (51). Similar data for serotypes 2-4 would be of considerable value in complementing the information available from Mabs studies so that more meaningful relationships within the lyssaviruses could be established.

PATHOGENESIS

In an elegant study of the comparative pathogenesis of rabies and rabies-like viruses (52,53), young hamsters were used as a model host system for street, Lagos bat, and Mokola virus infections. All aspects of viral pathogenesis in the CNS and in the organs involved via subsequent centrifugal virus spread were remarkably similar. Only in the cerebellum did fluorescent patterns vary: street virus was Purkinje-tropic whereas Lagos bat and Mokola viruses invaded all cerebellar layers.

A rabies-like illness preceded death when rhesus monkeys and dogs were inoculated intracerebrally with Lagos bat virus (54). In the first of several cases of Lagos bat virus infections in South Africa, the unidentified bat (probably an *Epomorphorus*) was found sick with a rabies-like disease (10,11). High temperature and drowsiness were features of 2 cases of Mokola virus infection in children, the second of whom died. At autopsy, cytoplasmic inclusion bodies seen in neurons were quite different in size and appearance from the Negri bodies of classical rabies virus infection (15).

Common signs in the cats which died of Mokola virus infection in Zimbabwe (55) were hypersensitivity to stimuli and muscle fasciculations, which led in some cases to initial diagnoses of insecticide poisoning or hypocalcemic tetany. Incoordination followed by paralysis sometimes occurred. Histological examination of the brains revealed unusually extensive meningoencephalitis, and although a few doubtful intracytoplasmic inclusions were found in the thalamic region of some of the brains, in general no Negri bodies were seen. Two genets, a mongoose, and a jackal were experimentally infected, and all showed

symptoms similar to those seen in naturally infected cats (55). Although 1 of 4 shrews from which Mokola virus was isolated was found dead near the laboratory at Ibadan (13), the other 3 were apparently healthy.

Remarkably (for a lyssavirus), Mokola virus is said to be both neurotropic and viscerotropic. Wild shrews inoculated with Mokola virus and which showed signs of illness were able to transmit the disease to mice by biting; some shrews died of Mokola virus infection after eating infected mice. Clinical signs of disease in shrews included a tendency to save food instead of eating it, and some became restless, followed by flaccid paralysis. Others became aggressive but less effective in attack. There were marked histological changes in the brain, with lesions in the salivary glands, but neither intranuclear nor cytoplasmic inclusion bodies were seen (52).

In the African case of human death from Duvenhage virus infection, clinical disease and post-mortem findings were consistent with a rabies virus infection (19). Electron microscopic studies of tissues from mice infected with virus from this case revealed budding upon endoplasmic reticulum and plasma membranes of brain neurons, resembling laboratory or fixed virus rather than street virus infection (19,57). In 1985, a human death from bat rabies virus infection occurred in Finland (33) and 2 deaths were reported from the U.S.S.R. (30) (see above).

Although Duvenhage virus infection can cause sickness and death in bats in Europe, it is not always possible to distinguish infected from healthy bats: 1 of 7 apparently healthy bats which flew into a trap-net was infected (P. Grauballe, personal communication).

SEROLOGICAL RELATIONSHIPS AND CROSS-PROTECTION STUDIES

Most national laboratories now use immunofluorescent techniques for rabies diagnosis, but occasionally difficulties are encountered that may lead to a suspicion of strain differences. Even when a fluorescent polyclonal antibody conjugate was used at twice its normal working strength on suckling mouse brain impressions, staining was poor with the rabies-related viruses although bright with classical viruses (57). At the Central Veterinary Laboratory, following passage of the bat virus from Finland, mouse brain impressions were recorded as

negative when a commercially prepared polyclonal conjugate was used, but positive when a similarly prepared appropriate monoclonal conjugate was used (A. King, unpublished data). Interestingly, in the original demonstration of this virus, antigen was detected in a brain smear by staining with a monoclonal conjugate (33).

Indeed, polyclonal antisera, with which the earlier epidemiological surveys were made and the various interrelationships within the lyssavirus group established, have now been largely replaced by the use of Mabs. These latter fall into 2 groups, directed either against specific sites on the nucleocapsid (N or NS proteins) or on the surface glycoprotein (G) of the virion, and it is now possible to show varying degrees of homology and heterogeneity, not only between serotypes but also between strains within serotypes (58-61).

Using Mab-Ns, serotype 2, 3 and 4 viruses can be clearly distinguished from each other (Table 3). Minor differences exist within serotypes 2 and 3, but the differences between the members of serotype 4 are more striking. African Duvenhage viruses (DUV1/2) are clearly distinguishable from European viruses (DUV3-8) and, as with the serotype 1 bat viruses in the U.S.A. (see Smith and Baer, this volume) Mab-Ns can be used to differentiate virus isolates from different geographical areas of Europe.

Using Mab-Gs, serotypes can also be clearly distinguished, but no differences are discernible within serotype 3 viruses (Table 4). Differences between the African and German Duvenhage G proteins are less pronounced than with those of the N antigens; nevertheless, the viruses can be distinguished from each other and from Duvenhage (Poland) and Duvenhage (Denmark) viruses.

It has thus become obvious that the concept of a group antigen, the N protein of the nucleocapsid (63), was an oversimplification, and indeed there is so much diversity between the antigenic structure of the N proteins that it is often easier to differentiate between viruses by using Mab-Ns than by Mab-Gs.

It has been shown that, while mice immunized with standard rabies vaccines are fully protected against homologous virus challenge and against street viruses that share several antigenic determinants, they are poorly protected against challenge with viruses of only a limited

Table 3. Indirect Immunofluorescence Using 37 Wistar Institute Mab-Ns on Fixed Infected Cultures

Mab-N number[a]	LAG	MOK 1	MOK 2	MOK 3	MOK 5	DUV 1/2	DUV 3/4/5	DUV 6	DUV 7	DUV 8
721-2	ø	ø	ø	ø	ø	ø	ø	ø	ø	+
377-7	ø	ø	ø	ø	ø	ø	o	+	o	o
801-1	ø	ø	+	o	o	ø	+	ø	ø	ø
803-6	+	ø	ø	+	+	+	+	ø	+	ø
806-1	ø	ø	o	+	+	ø	ø	+	ø	ø
120-2	ø	ø	ø	ø	ø	ø	ø	+	ø	ø
209-1	ø	ø	ø	ø	ø	ø	ø	+	ø	ø
229-1	ø	ø	ø	ø	ø	ø	ø	+	ø	+
237-3	ø	+	+	+	+	ø	ø	+	ø	ø
239-1	ø	+	+	+	+	ø	+	o	ø	ø
590-2	ø	ø	ø	ø	ø	ø	ø	o	ø	+
364-1	ø	ø	ø	ø	ø	+	ø	ø	ø	+
805-3	+	o	o	ø	ø	+	ø	+	+	+
515-3	ø	ø	ø	ø	ø	ø	+	+	+	+
206-1	ø	ø	ø	ø	ø	ø	ø	ø	ø	+
222-9	+	+	+	+	+	ø	ø	ø	ø	+
703-8	+	+	+	+	o	ø	o	ø	ø	o
104-4	+	+	+	+	+	o	o	+	+	+
111-1	+	+	+	+	+	+	+	+	+	o
422-5	+	+	+	+	+	o	ø	ø	ø	ø
102-27	ø	ø	ø	ø	ø	ø	ø	ø	ø	+
802-2	o	o	o	o	o	+	+	+	+	+
807-5	ø	+	+	ø	+	ø	ø	ø	ø	+
714-3	ø	ø	ø	ø	ø	ø	+	+	+	o
818-5	ø	ø	ø	ø	ø	ø	ø	ø	ø	+

[a] In addition to the 25 Mab-Ns listed, of the total 37 used by the Wistar Institute, Philadelphia, 3 (701-5, 715-3 and 103-7) were negative and 9 (808-2, 816-1, 502-2, 804-9, 111-1, 389-1, 1403-4, 817-5 and 822-7) were positive with all the above viruses (data not shown).

Symbols: + = positive reaction; o = variable reaction; ø = negative reaction. MOK = Mokola virus; LAG = Lagos bat virus; DUV = Duvenhage virus.

Virus origins: LAG, Nigeria; MOK1, Nigeria; MOK2, Cameroon; MOK3, Central African Republic; MOK5, Zimbabwe; DUV1/2, Africa; DUV3/4/5, Federal Republic of Germany; DUV6, Poland; DUV7, Denmark; and DUV8, Finland.

(Adapted from ref. 62).

degree of antigenic homology with the vaccine virus (64). It is also worth remembering that the dog infected with Mokola virus in Zimbabwe (see above) had been immunized with a classical rabies vaccine (17). In 1985, Wiktor (65) showed that the percentage of epitopes shared by the rabies virus used to prepare HDCV (Human Diploid Cell Vaccine; Wistar

Table 4. Neutralization Tests (RFFIT) using 41 Wistar Institute Mab-Gs

Mab-G number[a]	LAG	MOK 1/2/3/5	DUV 1/2	DUV 3/4/5	DUV 6	DUV 7
1119-14	ø	ø	ø	ø	+	ø
1107-1	ø	ø	+	+	+	ø
101-1	ø	ø	ø	ø	ø	+
1121-2	ø	ø	ø	ø	ø	+
1112-1	ø	ø	+	+	+	ø
613-2	ø	ø	+	+	+	ø
194-2	ø	ø	+	ø	ø	ø
523-11	+	ø	ø	ø	ø	ø
1105-3	+	ø	ø	ø	ø	ø
1113-1	ø	ø	ø	ø	ø	+
1122-3	ø	ø	ø	ø	+	ø
718-4	+	ø	ø	ø	ø	ø
1109-3	ø	ø	+	+	+	ø
1114-2	ø	ø	+	+	+	+
110-3	ø	ø	ø	ø	ø	+
1103-4	ø	ø	ø	ø	+	ø
1108-1	+	ø	ø	ø	ø	ø
1409-7	+	+	ø	+	+	+
176-2	ø	ø	ø	ø	+	ø
422-2	ø	+	ø	ø	ø	ø

[a] In addition to the 20 Mab-Gs listed, of 41 Mab-Gs used by the Wistar Institute, Philadelphia, 21 (509-6, 231-2, 228-8, 162-3, 116-1, 1111-1, 1117-8, 240-3, 719-3, 226-1, 248-8, 507-1, 120-6, 127-5, 904-4, 1118-6, 1120-1, 193-2, 504-1, 508-9 and 419-2) did not neutralize the above viruses (data not shown).

Symbols: + = virus neutralization; ø = no neutralization.

(Adapted from ref. 62).

Institute) and a selection of street viruses from the Americas, western Europe, and southern Africa varied from 44% for some viruses of bat origin to 100% for viruses from European foxes and Argentinean dogs, and from 64-100% for viruses from human cases in the U.S.A.: that is to say, classical rabies vaccines could be expected to protect both animals and man from infection by these serotype 1 viruses. In marked contrast, Duvenhage virus shared only 34% of its antigenic determinants with the rabies vaccine virus, and there were no common determinants between the latter and Mokola or Lagos bat viruses. As first pointed out by Shope and Tignor (66), there may be a case for producing polyvalent vaccines for use in certain parts of the world.

Schneider, in experiments quoted by Lumio *et al.* (33) showed that another classical rabies vaccine (suckling mouse brain; see Bunn, this volume) also gave poor protection in mice against the Hamburg and Stade viruses. Similar results were reported by Schneider and Meyer (67). However, as Wunner (48) has commented, this correlation of antigenicity between vaccine strains, challenge virus and the outcome of the infection may not hold in other species.

In view of the fact that Duvenhage virus now appears to be endemic among bats of northern and western Europe, the question whether vaccines prepared against classical strains will protect against challenge with bat virus has been re-examined by Bitsch and coworkers (34). These authors, in contrast to earlier workers, showed that both human and animal vaccines would protect mice against challenge with the Danish bat virus (DBV) and, most importantly, that human rabies immunoglobulin (HRIG) would also neutralize DBV. The latter observation has been confirmed by Lafon *et al.* (68) who undertook for the W.H.O. the examination of sera from immunized volunteers who had been given 1 of 5 high potency commercially-available vaccines using the newly proposed abbreviated schedule for post-exposure treatment. They also examined 5 different preparations of HRIG currently available in Europe in the same type of neutralization test (RFFIT; see Campbell and Barton, this volume) (69,70). All had high neutralizing antibody titers against both standard CVS and DBV. This satisfactory result led them to the conclusion that in cases of human contamination by an infected bat in Europe, post-exposure treatment with rabies vaccine and HRIG should be administered according to W.H.O. recommendations as for contamination by rabid terrestrial animals. But that is not the end of the story. In our laboratory, we have found that HRIG prepared in the U.K. from the sera of vaccinees who had received HDCV neutralized serotypes 2-4 far less efficiently than serotype 1 (A. King, unpublished data). Furthermore, Dietzschold *et al.* (71) have shown that 2 groups of mice given vaccines of different virus origins both produced high titers of antibody, but only 1 group resisted challenge with the serotype 4 bat virus from Poland.

DISCUSSION

Among the diseases of viral origin, rabies is unique in its distribution and in its range of victims since it can apparently afflict all warm-blooded animals. Rabies virus has particular affinities for the central nervous system and for mucus-secreting glandular tissue, creating the conditions for the spread of the virus by the injection of infectious saliva as the tendency to bite is increased (see Charlton, this volume).

Rabies in dogs has been known for centuries, and according to Johnson (72), "knowing that the dog is an aberrant host of the virus, this is a remarkable example of the persistence of basic genetic characters of a virus over a period of more than 2,000 years." It is only in the second half of this century that rabies viruses with properties so distinctive that they can be diagnosed by serological means have been discovered, and only within the last decade that laboratory tools have been developed which allow exploration of the genetic characters of the viruses. These tools reveal that in the vast majority of terrestrial species, the basic genetic characters of the virus are retained.

The Chiroptera are among the most ancient of mammals, and the single characteristic that separates them from all other mammals is their ability to fly. Almost all of the bat families inhabit the tropics, and the Rhinolophidae, Vespertilionidae and Molossidae are also found in temperate zones, within which they have developed the abilities to hibernate and to migrate. Rabies in insectivorous bats is prevalent throughout the U.S.A. Monoclonal antibody studies of the rabies viruses within these bats reveal differences between them and the viruses isolated from terrestrial animals in the same geographical area, but these differences are not sufficient to warrant separation from them on serological grounds. Rabies viruses of bat origin are detected in terrestrial animals, but these "spill-over" events are thought to be rare.

It is remarkable that, almost at the first attempt, Boulger and Porterfield should isolate the very virus they were looking for - rabies in a bat. Lagos bat virus appears to have a wide distribut ion within the African continent, though it does not appear to affect bats

in large numbers. Recently, the virus has been isolated from 2 cats (1 in Zimbabwe, 1 in South Africa; A. King, unpublished data) - further evidence that "spill-over" of bat rabies can occur and that when it does it can cause a lethal infection.

The insectivorous shrew genus *Crocidura*, from which Mokola virus was first isolated, inhabits Africa, much of Europe, and some parts of Asia. Shrews are fearless, aggressive, and voracious, and when disturbed they may assume a position in which they crouch on the ground with raised head and bared teeth, while emitting a single metallic squeak. Perhaps it is this attitude that commands both fear and respect from the inhabitants of Ibadan (14) where the first virus isolation was made. The animals usually manage to inflict a decisive head wound with the initial onslaught on prey, and in captivity, when they kill, the brain of the victim is always eaten first (73). Evidence that Mokola virus in naturally infected shrews is viscerotropic (which is unusual for a rabies virus), is equivocal. In 3 of 4 isolations made from non-nervous tissue pools, the brain was not examined, and in only 1 case was virus isolated from a liver and spleen pool but not recovered from the brain (13). Isolation of Mokola virus from the brain of a rodent has occurred once only. *Lophuromys* are insectivorous rodents of Africa which are found in a variety of habitats, including bushy country and forests. No other Mokola virus isolations were made in over 3,500 rodents examined in the same area over a 20 year period (18). Le Gonidec and coworkers considered whether or not Mokola virus may also have an arthropod host (16).

It is interesting that the 2 cases of Mokola virus infection in children described by Familusi and coworkers (14,15) presented neurological symptoms which were not, however, suggestive of rabies. This finding would support the view that Mokola virus may indeed be neurotropic as well as viscerotropic, but followed an evolutionary path different from viruses of serotypes 1, 2 and 4.

On present evidence within Africa, Duvenhage virus has a more limited geographical spread than Lagos bat virus, and does not infect large numbers of bats. There has been to date only 1 isolation of Duvenhage virus from a bat in South Africa, despite the fact that the initial isolate in 1970 came from a man who had been bitten by a bat

before his fatal illness. Foggin, who recently examined 1,500 bats captured in Zimbabwe, found that only 1 was infected with Duvenhage virus and none with Lagos bat virus (C.M. Foggin, unpublished data).

In marked contrast is the incidence of Duvenhage virus in Europe, where by the end of 1987 it had been identified in nearly 300 bats from 8 different countries (Fig. 2). The index case in Hamburg (25) predated that of South Africa by some 15 years, and the virus is not only persisting but is well-established among European bats. Thus the suggestion of a recent casual introduction of the disease from bats imported by ship from Africa (67) can be discounted, and a different reason for its presence should be sought. Serotine bats (which also inhabit Africa) appear to be the principal victims of the disease in Europe, although at least 6 other bat species have been implicated. In Finland, however, serotine bats have never been identified (33). Remarkably, the first indication of disease in bats in each of the European countries affected was by accidental human involvement.

Perhaps the disease has been present in Europe for many years, but it has been those incidents coupled with a current interest in bats that has encouraged the search for infection. It was the occurrence of similar incidents with humans in the U.S.A. that initiated the search for rabies within the bats of North America.

In Europe, as in North America, rabies infection in the bat population appears to be independent of that maintained within the terrestrial wildlife (principally foxes in Europe, foxes, skunks and raccoons in North America). Present evidence suggests that in Europe, a far higher proportion of bats may be infected than is the case in North America. However, in Europe, unlike in North America, bats are infected not with classical rabies (serotype 1) but with what was regarded as the exotic serotype 4, Duvenhage virus.

Rabies-related viruses *in vitro* show remarkable phenotypic plasticity (74). It is therefore perhaps not surprising that, among the isolates of serotypes 2-4, differences in the epitopic maps are so readily demonstrable (Tables 3 and 4). The bat viruses of North America (serotype 1) show a restricted degree of epitope sharing with the viruses from terrestrial animals of that continent (see Smith and Baer, this volume). Perhaps there are as yet unidentified features in bats

Figure 2. Primary isolations of serotype 4 viruses in Europe, by country. 1: F.R.G.; 2: G.D.R.; 3: U.S.S.R.; 4: Poland; 5: Denmark; 6: Finland; 7: Netherlands; 8: Spain.

which are absent in terrestrial animals but which provide greater opportunity for virus diversification.

It may be asked what has caused the sudden upsurge of Duvenhage viruses within Europe? In addition, what of the isolate from Finland which appears in our hands and others (J.S. Smith, personal communication) to be somewhat different from either the classical rabies or serotype 4 viruses? Clearly, the European bat rabies situation is arousing interest among public health authorities, and renewing interest among virologists who, at a fundamental level, wish to study the evolution of viral diseases (see Tordo and Poch, this volume).

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9

DIAGNOSIS OF RABIES INFECTION

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INTRODUCTION

In recent years there have been advances in cell culture technology, immunochemical procedures, and the use of peripheral nervous and non-nervous tissues for rabies diagnosis. Since the advent of monoclonal antibodies directed against various components of the rabies virion and their use to distinguish different rabies and/or rabies-like strains, it has become increasingly apparent that many of the biological curios noted in the past are probably valid expressions of different strains of rabies. It is no longer correct to say that all rabies virus isolates behave in a similar manner or that the characteristics of street strains can be inferred from the study of fixed strains. This is becoming evident in diagnostic situations. The laboratory worker must always be on guard for the unusual or altered reaction. We hope to address some of these aberrations in this review.

The principal routine diagnostic procedures in most industrialized countries consist of immunofluorescent staining of brain tissue and inoculation of suspect tissue into mice or cell cultures. However, for a variety of climatic or socio-economic reasons, different procedures may be used in different laboratories. Not all diagnostic laboratories are equipped with fluorescence microscopes, cryostats or CO_2 incubators or have access to laboratory animals. In preparing Table 1, we have attempted to present a choice of techniques suitable for different situations. The listing in Table 1 is not necessarily complete but hopefully contains the publications in which the particular techniques are more fully described.

We have relied heavily upon experience gained by personnel of the Animal Diseases Research Institute, Nepean (ADRI). During the twenty

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Table 1. Selected References Containing Techniques for Rabies Diagnosis

Tissue	Fresh	Fixed
Brain (CNS)	FAT*: 1-4,6,93,147, 148	FAT (formalin): 24,29,31, 32,34,36,37
	FAT (frozen sections): 71,106,113,131	FAT (acetone): 149,150
	MIT: 4,41-43,138,148	ELR: 90,91,93,95-98,151
	TC: 57,58,63,65-68,70	EM: 57,71,94,98,106,113,115
	HIST: 40,138,148,152	
	ELR: 90,99	
	EM: 112,153	
Salivary gland	FAT (frozen section): 106,131,154	EM: 106
Saliva	TC: 56,117	
	MIT: 48,56,121,125	
Cornea	FAT: 130,132,138,155	
Skin	FAT: 138-141,143	

* FAT = fluorescent antibody staining; MIT = mouse inoculation; TC = cell culture; HIST = histological staining; ELR = enzyme-linked reactions; EM = electron microscopy.

year period between 1966 and 1986, some 150,000 brain or nervous tissue specimens have been submitted for rabies diagnosis and the number of annual submissions is now approaching 13,000. With a staff of 5 devoted almost exclusively to routine rabies diagnosis, the procedures and techniques used must be both reliable and adaptable to the examination of large numbers of specimens on a daily basis. Although several techniques have been described in the literature and are acceptable as diagnostic procedures, some are impractical in laboratories burdened by large numbers of submissions. We have included some of our procedures which we have found to produce satisfactory results (See Appendix).

The histopathological aspects of rabies viral infection are described elsewhere in this book (Charlton), as are the serological diagnoses (Campbell and Barton).

IMMUNOFLUORESCENCE

The fluorescent rabies antibody (FA) staining technique used on impressions/smears of brain or nervous tissue was developed by Goldwasser and Kissling (1). This technique is now the standard primary test where fluorescence microscopy is available and has been well described by Kissling (2) and Dean and Abelseth (3). Velleca and Forrester (4) have outlined the principles of fluorescence microscopy and Emmons and Riggs (5) have described the application of immunofluorescence to diagnostic virology. The main requirements for the use of this technique include a conjugate of high quality, a suitable microscope and well-trained personnel.

Labelled anti-rabies antibodies can be prepared against (i) whole rabies virions in tissue suspensions (2,3,6); (ii) purified virus suspensions to increase the immunogenicity and purity of the antisera (7,8); (iii) sub-virion components such as the ribonucleoprotein (9); or (iv) as a mixture of monoclonal antibodies produced from hybridoma cultures (Centocor Inc., Malvern, PA, U.S.A.; Pasteur Institute, Paris, France). This latter method reflects recent advances in immunology (10) which produce a staining conjugate of greater specificity.

All conjugated antisera require stringent testing and evaluation to ensure specificity as well as uniformity of reaction (3,5,11,12). These quality assurance procedures help to ensure the highest quality of reagents (13) and their consistent use in different laboratories (11,14). The quality of reagents such as acetone must be verified before being put into routine use, since contamination with some chemicals can seriously affect results of the FA test (ADRI unpublished, 15). The pH of the water used for rinsing of the stained slides should be checked periodically to ensure that extremes in values do not occur. After a distilled water reservoir had been cleansed and flushed with acid, the staining intensity was much reduced and alternative supplies of water were required before the situation was rectified (ADRI unpublished). Durham et al. (16) found that mounting media with

a pH of 8.5 was superior for use with modern fluorescein conjugates to produce good fluorescence and to maintain brilliance over a short period of time. Fading of the staining reaction can be retarded through the use of polyvinyl alcohol mounting medium containing paraphenylenediamine or n-propyl gallate (17).

Impressions or smears of nervous tissues contain infectious virus (18,19) as does the virus suspension used to absorb the fluorescein-tagged antibodies contained in the staining suspension. Virus in the slides and the staining suspensions can be inactivated with heat (20), beta-propiolactone (20,21) or gamma radiation (22) to eliminate the hazard of working with infectious virus.

Formalin-fixed brain tissue may be stained by the FA technique, but the tissue requires digestion with an enzyme such as trypsin and/or pepsin to unmask the immunoreactive sites (23-37). This modified FA test is suitable for retrospective studies on tissues which have been formalin-fixed and stored for some time (38). Sensitivity of 90-100% has been reported (23,31,36,37) as compared to results from fresh tissues. Digestion with trypsin for longer than 60 min may result in false negatives (30). This technique should not replace the use of fresh tissue smears unless absolutely necessary.

In this laboratory we have combined the methods of Umoh and Blenden (37) and Barnard and Voges (24) for staining formalin-fixed brain tissue. We have obtained reasonable brilliance of fluorescence by including 0.3% sodium citrate in the trypsin solution and increasing the concentration of the conjugated antibodies. The fluorescence appears as inclusions or aggregates of antigen in the cytoplasm of neurons throughout the section. For the most part only the larger aggregations or inclusions are easily identifiable, the small "dust-like" particles, which are easily found in fresh tissues, do not stain well.

ANIMAL INOCULATION

The isolation of rabies virus by intracerebral inoculation of animals is feasible in several laboratory animal species; rabbit, guinea pigs, hamsters or mice. Suckling mice are more susceptible to some strains of street rabies virus than are older mice (39) but for

diagnostic purposes newly weaned mice are used. This technique has been described by Atanasiu (40), and Koprowski (41).

Experimental mice are routinely inoculated intracerebrally with a 10% suspension of brain tissue and observed for mortality during a 30 day period. This 30 day observation period may be reduced if sufficient numbers of mice are inoculated so that one mouse can be sacrificed per day post-inoculation and the brain examined by the FA test. In this way a diagnosis of rabies may be made well in advance of the appearance of clinical signs and/or death (42-44).

Lodmell (45), Lodmell and Chesebro (46) and Blancou *et al.* (47) have studied the relative susceptibility of various strains of experimental mice to a single street rabies virus strain. Although different mouse strains varied in susceptibility following intraperitoneal inoculation of virus, no apparent differences were noted following intracerebral inoculation (45). However, not all street rabies virus strains will infect the same mouse strain and produce clinical signs and/or mortality in a similar manner. Baer *et al.* (48), Larghi and Diaz (49) and Skalka (50) demonstrated that some virus isolates were more pathogenic than others. Webster *et al.* (51) have demonstrated that virus from the brain and salivary gland of the same animal may produce strikingly different mortality periods in the same mouse strain. Differences in mortality rates may also be influenced by the available virus/neutralizing antibody ratio in the tissue suspensions used as inocula, either in brain (52) or salivary gland (53,54).

High ambient temperatures within the animal facilities may delay the onset of clinical signs, decrease mortality and increase the frequency of abortive infection (55).

CELL CULTURE ISOLATION TECHNIQUES

Fixed rabies virus can be grown in various cell cultures and these systems have been used for numerous experimental studies (see Chapter 3). The successful infection of cell cultures with street rabies virus has been more difficult and only relatively recently have techniques been developed whereby these strains can be isolated consistently from suspect animal tissues.

Initial attempts at isolating street strains were made using baby hamster kidney (BHK-21) cell cultures (56-60). Various other cell types have been used including CER cells (61,62,63), a variety of skunk, dog and raccoon cell cultures (63) and a murine neuroblastoma (NA) cell line (57,61,63-68). This latter cell type has been shown to be more susceptible to infection by street rabies virus than others and is now being used at laboratories where the mouse inoculation test has been replaced by a tissue culture test. A test system suitable for the examination of some 5,000-6,000 FA negative specimens annually is presented in the Appendix.

Numerous studies have compared the infection rate of BHK and NA cells with street rabies virus and have found that NA cells are more susceptible to infection (63,66-68). This difference in susceptibility may be related to viral strain differences as well as cell type. Comparisons (51) of 2 antigenically different rabies virus strains found in Canadian terrestrial mammals illustrate this feature. The virus strain common to skunks and foxes in eastern and northern Canada grows well on NA cells and poorly on BHK cells during initial isolation attempts from brain tissue. Passage of this virus through 4 sub-cultures of BHK cells changes the growth characteristics such that this virus can then be readily replicated in BHK cells. The skunk rabies virus strain found in western Canada grows equally well on both cell types during initial isolation (51).

Although most street isolates from brain tissues grow readily in neuroblastoma cells, some, because of the low viral content in the original host tissue, may infect only a small percentage of cells on initial isolation attempts (66,68). A number of such isolates have been inoculated into NA cell cultures and replicates were examined daily for a 4-day period (ADRI unpublished). Some isolates grew well but 7 of 10 were negative when examined at 24 hrs and 4 of these still showed less than 5% cell infection at 48 hrs post inoculation. All were positive at 4 days. Most specimens which are rabies negative by the fluorescent antibody staining test and subsequently proven rabies positive by other procedures usually have only very small amounts of virus in the original tissues and it is these specimens for which a 4 day incubation period is required. During the year following the

adoption of a tissue culture test at this laboratory (68), some 5700 specimens (previously FA negative) were tested by this method. Of these, 12 were proven rabies positive. With the exception of 1 of these positive specimens, the upper limit of cell infection at 4 days post infection was 15%; most were in the 1-5% range. Fast (69) reported on a case where 3 canine litter-mates, suspected of having been in contact with a skunk, were all diagnosed as rabies positive by cell culture procedures but only 1 was rabies positive by initial immunofluorescence staining. Barrat and Picard (64) also found that incubation of cultures for only 24 hrs failed to demonstrate rabies virus in specimens which were rabies positive by the mouse inoculation test.

For any test to succeed in replacing the standard mouse inoculation test (41) it must be at least as sensitive at demonstrating rabies virus. BHK cells are equally as sensitive as mice (56,58, 67) while NA cells are much more sensitive to infection by street virus than are mice (61,65-68). This is especially evident when suspensions containing very small amounts of virus are compared. It was demonstrated that, of 159 brain suspensions diluted to contain only sufficient virus to infect 1-5% of neuroblastoma cells, 87 (55%) did not kill mice (68). As noted above most specimens that are rabies-negative by the FA test and subsequently proven positive by some other test usually have only small amounts of infectious virus in the tissue.

Co-cultivation techniques using BHK, NA or rat glioma cells have been attempted with variable results (57,70,71). As a routine test for use in field cases, these techniques have so far been somewhat cumbersome and susceptible to contamination.

Cell cultures are susceptible to contamination and lysis by bacteria, fungi, other viruses, and toxins found in suspect animal tissues. The use of appropriate antibiotics in the culture media greatly improves the resistance of cultures to bacteria (72). We have centrifuged and/or filtered obviously contaminated brain tissue suspensions in an attempt to remove bacteria and fungi. However, using the neuroblastoma cell culture isolation procedure (68) it was found that the use of a 0.22 μm Millipore filter reduced the amount of virus in a suspension by 50-100% depending upon the original concentration (ADRI

unpublished). Centrifugation produced mixed results with respect to bacterial contamination and sometimes resulted in a decrease in the amount of virus in the inoculum. Grinding brain tissue in physiological saline containing 500 IU penicillin G and 2 mg streptomycin sulfate/ml, and allowing this suspension to settle at 4°C for at least 1 hr prior to withdrawing the clear supernatant layer for use in the cell culture test, has greatly reduced the number of cultures rendered unfit because of bacterial contamination.

The cells in plastic microtitration plates can be readily stained with an antirabies antibody/fluorescein conjugate following fixation with 75-80% acetone (T.J. Wiktor, personal communication). Fixation of these plates with 10% formalin substantially reduces the brilliance of the fluorescence to such an extent that small dust-like particles and/or small amounts of antigen may not be visible (ADRI unpublished).

Saliva samples have been used (56,73) and can indicate rabies infection under certain conditions (see also below). The infection of BHK-21 cell cultures with saliva specimens often results in cell lysis at low saliva dilutions due to bacterial or toxin contamination; higher dilutions may not contain virus. Neuroblastoma cell cultures generally tend to be more resistant to cell lysis in this case.

MONOCLONAL ANTIBODY PROFILES

Monoclonal rabies antibodies were first developed by Wiktor and coworkers (10) for the detection of antigenic differences between rabies and/or rabies-like virus strains. This original panel of antibodies has been supplemented with others and it is now possible to characterize many of the field strains of rabies and rabies-like viruses from various parts of the world and to separate many of these from fixed rabies virus strains (see King and Crick; Smith and Baer, this volume).

Although the use of monoclonal antibodies has centered more on the epizootiological aspects of rabies, they can be useful in diagnostic situations. They have been recently used in an attempt to identify the virus strain involved in the occurrence of "rabies" in man (74) and bats (75) in Europe. Wiktor *et al.* (76) demonstrated that a virus which produced reduced fluorescence in brain smears of cats from

Zimbabwe when stained with ordinary polyclonal anti-rabies fluorescein conjugate (77) was in fact a rabies-related (Mokola) virus. Where wildlife vaccination programs are in progress (78,79), diagnostic laboratories may be asked to confirm the origin of virus in a given specimen; i.e., whether the infection was caused by a field or a vaccine strain. Vaccine-induced rabies has been implicated in several domestic animal infections and monoclonal antibodies have been used to confirm some of these (80-83).

Staining with anti-nucleocapsid monoclonal antibodies can be performed on brain smear/impression slides (10,84-87) or on infected cell cultures grown in 60-well HL-A Terasaki plates (60,76,85,88). Neutralization with anti-glycoprotein monoclonal antibodies is usually done with cell cultures (76,89).

ENZYME-LINKED REACTIONS

For those laboratories which do not have access to fluorescence microscopy, various techniques using immunoenzymatic reactions may be beneficial. Atanasiu *et al.* (90) proposed the use of an enzyme immunoassay for the detection of rabies antigen in tissue impressions. Since then, modifications have been developed for the detection of antigen in tissue sections and impressions (30,33,91-96). Fairly consistent results can now be obtained although most techniques are time-consuming and not readily applicable to routine diagnosis of large numbers of specimens. However, where formalin-fixed tissues are the only ones available, immunoenzymatic techniques offer an attractive alternative to immunofluorescent staining. Minamoto *et al.* (97) and Reville-Monsalve *et al.* (98) applied the technique to cell cultures and used both light and electron microscopy. The method (91) currently being used at this laboratory for the examination of formalin-fixed tissue is outlined in the Appendix.

Enzyme-linked immunosorbent assays have been developed for use in antibody determinations. Recently Perrin *et al.* (99) developed this technique to determine the presence of rabies antigen in tissue suspensions. This technique has been adapted for the testing of large numbers of suspect field submissions and compares favourably with immunofluorescence (100).

ELECTRON MICROSCOPY

The use of electron microscopy has enabled researchers to determine many of the physical characteristics of the rabies virion as well as events connected with the infection of, maturation in and release of virus from host cells. Much of this work has been reviewed by Matsumoto (101), Dierks (102) and Murphy and Harrison (103). The electron microscope has not been used to any extent in rabies diagnosis but has been recommended for use as a rapid diagnostic technique of viral infections (12). The general techniques involved in rapid diagnosis of viruses of medical and veterinary importance using the electron microscope have been well presented by Doane and Anderson (104) and Miller (105). While most studies have centered around infections with various fixed rabies strains, several have described the appearance of infection due to street virus strains (71,94,102, 106-115). Most of these studies have demonstrated virus in cells or tissues. Negative staining of virus suspensions has not been used to any extent in rabies diagnosis. As noted above, immunoperoxidase staining has been used on cell cultures infected with street virus and examined by electron microscopy (97,98).

TISSUES USED

Rabies antigen may be distributed unevenly throughout the brain especially in the early stages of the disease. Even when widespread, some areas contain greater amounts of antigen than others. Maserang and Leffingwell (116) discussed the impact of laboratory examination of only a single, small area of suspected tissue and recommended that more than one area be examined. Trimarchi *et al.* (117) demonstrated that the same virus suspension inoculated into four cats produced varying amounts of antigen within different areas of the CNS of individual animals and also between the 4 cats. Two horses which were proven to have had rabies showed little or no viral antigen in the brain; virus was demonstrated mainly in the spinal cord (118).

Rabies virus antigen can be found in a wide variety of tissues (119; see also Chapter 5). However, most of these tissues are either unsuitable or unavailable for routine diagnosis. Alternative tissues that can be used when only the head is available for diagnosis are the

salivary glands, cornea, skin, and tonsils. Fekadu *et al.* (71) demonstrated rabies virus in the tonsils of dogs. Virus has been identified in salivary glands or saliva (56,73,117,120-124). It has been noted that rabies virus may be present in the saliva of infected animals some considerable period of time before the onset of clinical signs (53, 121,125,126). However, depending upon the strain of rabies virus, the amount of virus isolated from saliva may vary over a period of time and may actually disappear before death of the animal (Rupprecht and Charlton, unpublished). This feature has also been noted in human infections (127-129).

The diagnosis of rabies in a living animal by fluorescent antibody staining of corneal impressions was first described by Schneider (130). Since then, the test has been evaluated and used in humans, dogs, herbivores and laboratory animals (122,131-137) and may provide a positive diagnosis. However, a negative staining reaction should not be interpreted as indicating a rabies-negative animal (138). In the majority of diagnostic situations, impressions are prepared in the field and then submitted to the laboratory. It has been our experience that such specimens may be received in a very poor condition (because of poor collection technique, improper transportation procedures, etc.) and be unsuitable for proper examination and diagnosis. Preliminary studies (ADRI unpublished) have indicated that fluids obtained following washing of eyes of post mortem specimens with a saline/ antibiotic solution may contain viable virus when tested by cell culture techniques. This procedure could be used when brain material is unavailable for testing.

Probably the best method for the ante-mortem diagnosis of rabies in animals and man is the examination of skin biopsy material. However, care must be taken when performing the biopsy that hair follicles are obtained and that the specimens are forwarded to the examining laboratory in good condition (139). Rabies virus has been demonstrated in the nerve network surrounding hair follicles and in epithelial cells by immunofluorescent staining on frozen sections of skin biopsies from mice, dogs, skunks, cattle and cats (140) and from skin biopsy material from humans (141). Wright (142) diagnosed a calf as rabid following the examination of skin biopsy material and this diagnosis was con-

firmed by immunofluorescent staining of brain tissue following death. Blenden *et al.* (139, 143), Umoh and Blenden (144) and Ciuchini *et al.* (145) examined a variety of tissues and hosts and proved the efficiency of this technique. Experimental mice examined sequentially during the course of disease were positive by skin biopsy some time before the onset of clinical signs and most continued to be rabies-positive until the termination of the experiments (140,143,145). Other animal hosts may not be found positive by skin biopsy during the greater part of the disease and may be positive only at or near death (146). Rabies virus antigen may be demonstrated in skin biopsies from humans at the onset of clinical signs and subsequently sporadically or not again (127,138) or infrequently at the onset of clinical signs and with increasing frequency to the termination of the disease (139,146). The site of inoculation may influence the reliability of this technique since Ciuchini *et al.* (145) found that approximately 70% of mice inoculated intracerebrally were rabies positive by skin biopsy while only approximately 36% were positive following intramuscular inoculation.

An interesting case presented to this laboratory illustrates the usefulness of examining any nervous tissue. A bovine had been slaughtered, butchered and frozen and was subsequently suspected of having had rabies. A piece of nerve dissected from a T-bone steak was proven by fluorescent antibody staining to contain rabies-specific antigen (ADRI unpublished).

Care must be exercised in interpreting negative results on tissues following long-term storage at low temperatures. Wachendörfer *et al.* (59) demonstrated loss of infectivity of brain tissue stored at -20°C. Unpublished data from this laboratory have indicated that storage of tissue suspensions for 6 months at -70°C can also result in a drastic reduction of viable virus, even in the presence of serum.

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APPENDIX

ROUTINE DIAGNOSTIC PROCEDURES USED AT ADRI NEPEAN

A. Fluorescent Antibody Staining

1. Remove portions of thalamus, pons, hippocampus and cerebellum from the brain and mix well on a paper towel.
2. Prepare 2 impression smears per microscope slide from this mixture. One slide is prepared of tissues from rodents, 2 slides from non-rodents with which there has been no human contact and 4 slides from non-rodents with which there has been human contact.
3. Air dry slides and fix in cold acetone at -20°C for 1-2 hours.
4. Following acetone fixation, air dry the impression slides and stain with a fluorescein-rabies antibody conjugate/ mouse brain suspension. Conjugate with normal mouse brain is applied to one impression and conjugate with rabies positive mouse brain to the other impression on each slide.
5. Control rabies positive impressions are also stained.
6. Incubate slides at 37°C in a humid chamber for 30 min.
7. Rinse slides in phosphate-buffered saline, pH 7.4, for 10 min.
8. Rinse briefly in distilled or tap water to remove excess salt.
9. Air dry and cover with coverslip using a glycerine mounting medium (50% glycerol buffered to pH 7.8).
10. Examine with a fluorescence microscope.
11. Frozen sections of skin biopsy tissue are mounted on microscope slides and stained as above.

B. Isolation of Rabies Street Virus in Neuroblastoma Cell Cultures

Tissue suspension:

1. Grind suspect brain tissue with a wooden popsicle stick on a paper towel and place brain tissue in a 17 x 100 mm sterile disposable tube. Add physiological saline containing penicillin and streptomycin to make a 10% (w/v) suspension and allow to settle for at least 1 hr at 4°C.

2. Prepare 10^{-1} dilution of this brain suspension using MEM-10[a] (=10^{-2} of original brain tissue).

Cell suspension:

1. Trypsinize stock neuroblastoma (NA) cells in T-25 flask and resuspend to give 3-5 x 10^5 cells/ml in MEM-10.
2. Just before use, add DEAE-dextran to a final concentration of 25 µg/ml.

Test:

1. Add 0.1 ml cell suspension to each of 4 wells in a 96-well microtitration plate. Repeat for each specimen to be tested.
2. Add 0.2 ml of tissue suspension to each of 4 wells and mix well.
3. Incubate at 35-36°C with 5% CO_2 for 4 days.
4. Prepare a positive (3 wells) and negative (2 wells) control SAS[b] plate *after* test plate has been prepared.
5. Following incubation, remove media from wells and wash once with phosphate-buffered saline (pH 7.4).
6. Add 75% cold acetone to each well and fix at RT for 30 min.
7. Shake out acetone and air-dry.
8. Add 1 drop of fluorescein conjugate/well and incubate at 37°C for 30 min.
9. Wash once with phosphate-buffered saline.
10. Counterstain with Evan's Blue (1:200) for 5 min and rinse once with phosphate-buffered saline.
11. Examine plate inverted on UV microscope using 10 x objective.

C. Examination of Formalin-fixed Tissues by Immunofluorescence

1. Paraffin-embedded tissues are sectioned at 5 um and floated onto microscope slides.
2. Place in oven at 60°C for 30 min.
3. Deparaffinize with 3 changes of xylene (2, 5 and 5 min).
4. Pass through 3 changes of ethanol (100%, 100%, 95%) for 2 min each.
5. Rinse 3 x 5 min changes in phosphate-buffered saline (pH 7.4).

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[a] Eagle's minimum essential medium supplemented with 10% fetal bovine serum, 10% tryptose phosphate broth, 2 mM L -glutamine and 50 µg/ml neomycin sulphate.

[b] A single plastic plate containing only 5 wells (Lab-Tek Division, Miles Laboratories, Inc.).

6. Digest in 0.25% trypsin in Sørenson's buffer (containing 0.3% sodium citrate and 0.6% sodium chloride) at pH 7.8 for 45-60 min at 37°C.
7. Wash 2 x 5 min changes in phosphate-buffered saline (pH 7.4).
8. Air dry and fix in acetone at -20°C for 60-120 min.
9. Air dry.
10. Stain with rabies antibody-fluorescein conjugate (2-3 x concentration used for staining brain impressions) for 45 min at 37°C.
11. Wash 2 x 10 min changes in phosphate-buffered saline.
12. Rinse in water, air dry and mount coverslip with buffered glycerol (pH 7.8).
13. Examine with a fluorescence microscope.

D. Peroxidase-antiperoxidase (PAP) Staining of Formalin-fixed Tissues

1. Fix tissue in 10% formalin (pH 5.3) buffered with Sørensen's phosphate buffer for 24 hrs.
2. Dehydrate in ethanol, clear in xylene, embed in paraffin (melting point 56-58°C).
3. Cut sections at 6 μm and float sections onto gelatin-coated slides.
4. Deparaffinize in 3 X 1 min changes of RT xylene.
5. Pass through 3 X 15 sec changes of 95% ethanol.
6. Hydrate in 0.05 M Tris-buffered saline (pH 7.5).
7. Expose sections to 0.3% H_2O_2 in 100% methanol for 30 min.
8. Rinse in distilled water.
9. Wash with 0.05 M Tris-buffered saline (pH 7.5) for 5 min.
10. Expose to 0.1% pepsin in 0.01 N HCl at 37°C for 30 min.
11. Rinse with Tris-buffered saline.
12. Expose to inactivated normal swine serum [diluted 1/20 in 0.05 M Tris-buffered saline (pH 7.5)] for 30 min.
13. Drain off serum but do not rinse.
14. Add primary antiserum (rabbit antirabies serum) at 37°C for 2 hrs.
15. Wash in Tris-buffered saline for 20 min.
16. Add link serum (swine antirabbit serum) for 30 min.
17. Wash in Tris-buffered saline for 20 min.
18. Add PAP complex (horseradish peroxidase and rabbit anti-horseradish peroxidase diluted 1/200 in Tris-buffered saline) for 30 min.
19. Wash in Tris-buffered saline for 20 min.

20. Add substrate (0.05% 3-3 diamino-benzidine hydrochloride in 0.05 M Tris-buffered saline with 0.01% H_2O_2)[c] for 3 min.
21. Wash with Tris-buffered saline for 20 min.
22. Counterstain in Gill's hematoxylin.
23. Dehydrate in ethanol, clear in xylene and mount in Permount.

E. Preparation of Rabies Nucleoprotein

(With kind permission of Dr. A. Wandeler, University of Bern, Switzerland.)

1. CVS-infected BHK 21/C13 cells are harvested (20-40, 175 ml flasks) by discarding the supernatant fluid, scraping the cells and resuspending these in a few ml of STE buffer (pH 7.4) (0.15 M sodium chloride, 0.01 M Tris-HCl and 0.001 M EDTA).
2. Freeze/thaw the cell suspension several times.
3. To 10 parts of cell suspension add 2 parts of 10% NP-40 (non-ionic detergent) in STE buffer and shake gently for 30 min at 4°C.
4. Add equal quantity of Arcton (trichloro-trifluoroethane) and mix gently for 30 min at 4°C.
5. Centrifuge for 10 min at 500 g.
6. Collect supernatant (discard interphase and bottom phase) and add NaCl to final molarity of 0.5 M.
7. Add 6% PEG 6000 (polyethyleneglycol) and stir 1-12 hrs at 4°C.
8. Centrifuge for 15 min at 500 g and discard supernatant.
9. Add a few ml of STE buffer and elute gently for 12-18 hrs at 4°C.
10. Centrifuge for 10 min at 500 g and harvest supernatant.
11. Re-elute sediment in a few ml STE buffer for 10 min, centrifuge for 10 min and harvest supernatant.
12. Pool supernatants from 10, 11.
13. Add CsCl (0.3866 g/ml supernatant) and centrifuge for 16 hrs at 100,000 g.
14. Harvest opaque band and dialyze against phosphate-buffered saline (pH 7.4) overnight.
15. Mix 1:1 with complete Freund's adjuvant and inject 1 ml each intraperitoneally and subcutaneously into goat (or rabbit).

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[c] Prepare and filter immediately before use.

F. Preparation of Fluorescein-tagged Antibodies

1. Collect serum of 3 weeks from goat (or rabbit) (see E above) and test for antibody titer. If titer (as tested by indirect immunofluorescence) is less than 1:100, revaccinate.
2. Add 1.0 ml saturated ammonium sulfate solution dropwise with constant stirring in an ice bath to each 1.5 ml serum. Continue stirring for 15 min.
3. Centrifuge at 3000 g for 20 min and discard supernatant fluid.
4. Resuspend sediment in 0.01 M phosphate buffered saline (PBS) until volume equals original volume of serum.
5. Repeat steps 2-4 until supernatant fluid remains clear.
6. Take up final sediment in 0.01 M PBS in half the original serum volume.
7. Remove ammonium sulfate by dialysis against 0.01 M PBS for 2-4 days. (Change dialysis bath twice daily).
8. Collect globulins, record total volume and determine total protein content by biuret reaction.
9. Add carbonate-bicarbonate buffer (pH 9) in an amount equal to 1/10 of total volume (see 8). Add 0.01 mg fluorescein isothiocyanate per mg protein. Stir overnight at 5°C.
10. Remove unreacted fluorescein by passing through a Sephadex G-25 column.
11. Test final conjugate to determine correct working dilution.
12. Test final conjugate against known rabies-positive tissues from a wide variety of animal species.

10

SERODIAGNOSIS OF RABIES: ANTIBODY TESTS

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INTRODUCTION

Rabies antibody tests are used primarily to assay the immune status of man and other animals following vaccination. They are of limited value in the detection of rabies-infected animals, since the immune responses following natural infection vary considerably, and antibodies may be produced only in the terminal stages, if at all (1,2).

The mouse neutralization test was introduced over half a century ago to measure rabies virus-neutralizing antibodies (3). It remains a widely accepted reference method. In the modern diagnostic laboratory, however, it has been extensively supplanted by more rapid and economical techniques. It is the purpose of this review to describe current techniques and to comment on their individual advantages and disadvantages. Some tests have been grouped under headings relating to the basic technique involved. It should be noted, however, that this classification scheme is quite arbitrary, and that certain tests could be considered under more than one heading.

THE MOUSE NEUTRALIZATION TEST

Originally developed by Webster and Dawson (3) to study the immunizing value of commercial antirabies vaccines, the serum-virus neutralization test in mice (MNT) remains the accepted reference method for all other antibody tests (4-6). In it, a constant amount of rabies challenge virus (e.g., 50-200 $MICLD_{50}$ (50% lethal dose by mouse intracerebral inoculation) of the challenge virus standard strain, CVS) is mixed with serial dilutions of the test serum. Following an incubation

period to permit virus neutralization (if antibodies are present), aliquots of each dilution are inoculated intracerebrally into weanling mice. If the serum dilution contains sufficient protective antibodies to neutralize the challenge virus dose, the animals will survive; if not, symptoms of rabies will develop and deaths will occur between 6 and 20 days post-inoculation. The testing of serial dilutions permits an estimate of antibody titer, the endpoint being related to the highest serum dilution that provides protection against the challenge. In terms of specificity this test remains unchallenged. However, it is expensive because of its requirement for live animals. It is also quite labor-intensive, and requires 3 weeks to complete.

THE PLAQUE NEUTRALIZATION TEST

The viral plaque assay, as introduced by Dulbecco in 1952 (7), involves the infection of susceptible cell monolayers with a limited number of virions, followed by addition of a nutrient semi-solid agar overlay. In a few days, replication of viral progeny stemming originally from single infected cells produce visible localized circular areas of cytopathic changes called plaques. In many cell cultures infected with rabies virus, however, no visible cytopathic effect is observed, although virus-infected areas can still be identified by their reduced ability to take up vital stains such as neutral red. Plaque formation by the HEP Flury virus strain was first reported by Yoshino and coworkers (8) in chick embryo monolayers; later systems utilized baby hamster kidney (BHK-21/13S) cell cultures suspended in agarose (9), BHK-21 cell monolayers with a Sephadex G-200 overlay (10), and porcine kidney cell monolayers under a carboxymethylcellulose overlay (11). (See also Crick and King, this volume).

By measuring the ability of a serum to inhibit viral plaque formation, the plaque assay can be used to determine antibody titers. In one method (12), suspensions of virus (e.g., 50-100 plaque-forming units (PFU)) and serial serum dilutions are incubated together for 90 minutes at 37°C, then spread over a thin, confluent layer of BHK-21 cells immobilized in nutrient agarose. After a 6 day incubation period the monolayers are stained with neutral red and the number of plaques at each serum dilution counted. A plot of plaque number versus serum

dilution is used to establish the serum antibody titre at the 50% plaque reduction endpoint.

IMMUNOFLUORESCENCE-BASED TESTS

Introduction of immunofluorescence into the diagnostic virology laboratory in the 1950s permitted the development of a number of *in vitro* tests for measuring rabies antibodies. Although they vary considerably in detail, these tests are based on the same detection principle: measurement of specific complexes involving antibody tagged with a fluorescent material or fluorochrome, such as the isothiocyanates of fluorescein and rhodamine. Molecules of these fluorochromes become excited when exposed to light of short wavelengths (long UV and violet-blue), and in reverting to their resting state, they emit light of a longer wavelength. This emitted light, or fluorescence, can be detected visually by incident light microscopy, or measured electronically in fluorometers (for reviews see refs. 13-15).

One problem associated with these techniques is that many biological materials, including cells and serum, produce autofluorescence that may result in high background levels with a consequent reduction in sensitivity. In many assays, however, advances in instrumentation and preparation of immunological reagents have minimized this factor. This is particularly the case in the technique of time-resolved fluorometry, which utilizes specialized instrumentation and fluorescent probes with long decay times; e.g., chelates of europium. Following a short excitation pulse, the long decay time permits measurement of specific fluorescence after the shorter-lived non-specific (background) fluorescence has disappeared. Although not yet reported for rabies, this technique has been utilized in the clinical diagnosis of respiratory viral infections (16).

The Indirect Fluorescent Rabies Antibody Test

In the indirect fluorescent rabies antibody (IFRA) test of Goldwasser and Kissling (17), glass slides with smears of infected mouse brain are incubated with the test serum. An anti-test species antibody conjugated with a fluorochrome is then added as indicator. Anti-rabies antibody present in the test serum will bind to the antigen in the rabies-infected mouse brain, and will in turn complex with the

fluorochrome-labelled anti-species (anti-globulin) antibody. The fluorescent complex can then be visualized under a microscope fitted with UV light optics. Slides incubated with antibody-negative serum samples will not show specific fluorescence.

This test can also be carried out with rabies-infected cell cultures replacing the infected mouse brain smears (Fig. 1). In both versions, the test measures binding antibody rather than specific neutralizing antibody, and since infected brain smears and virus-infected cells contain a preponderance of nucleocapsid (NC) antigen, it measures largely non-neutralizing anti-NC antibodies. This in itself would not be a major drawback if the levels of anti-NC antibodies reflected the levels of anti-G (neutralizing) antibodies; however, vaccines such as those prepared in duck embryo or in nervous tissue contain a preponderance of NC antigen. In such cases, discrepancies between IFRA and MNT results have been observed (18,19).

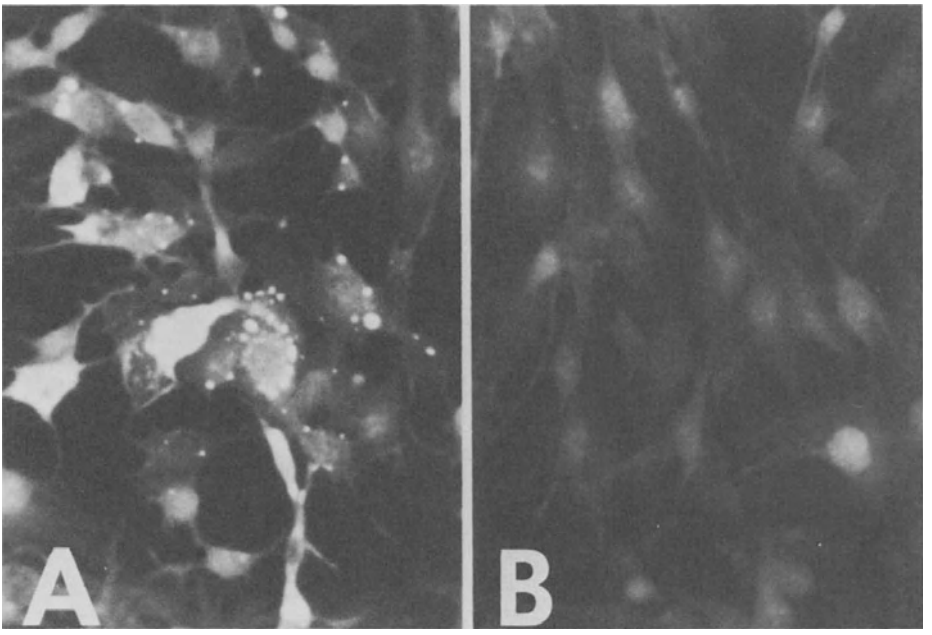

Figure 1. BHK-21 cell cultures stained with fluorescein-labelled anti-rabies globulin. A. Rabies-infected (ERA strain); B. Uninfected.

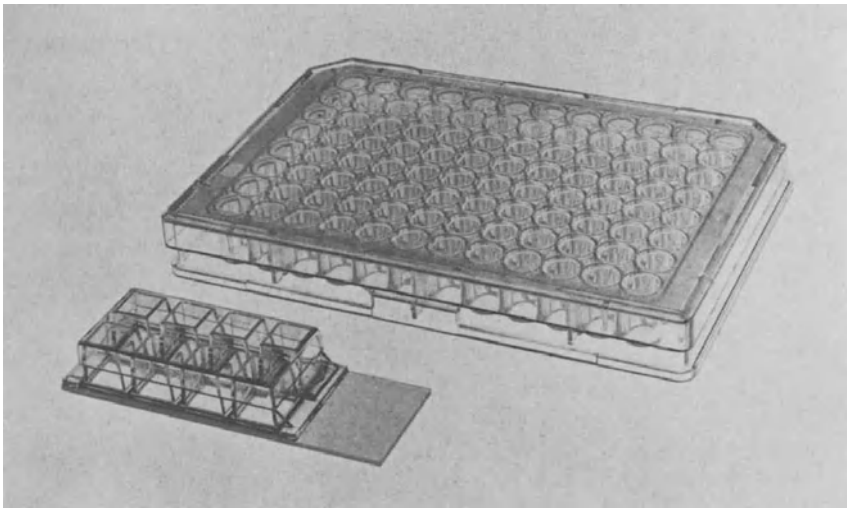

Figure 2. Top: 96-well microtitration plate (A/S Nunc, Kamstrup, Denmark, and other suppliers). Bottom: Lab-Tek tissue culture chamber slide (Miles Laboratories, Inc., Naperville, Illinois, U.S.A.).

The Rapid Fluorescent Focus Inhibition Test

By incubating test serum samples and challenge virus before addition to cell cultures, fluorescent antibody-based assays have been developed that measure specific neutralizing antibodies (20-22). Currently, the rapid fluorescent focus inhibition test (RFFIT) of Smith and coworkers (22) is probably the most widely-accepted alternative to the MNT. In this test, suspensions of BHK-21 cells are added to preincubated mixtures of test serum dilutions and challenge virus in 8-chamber Lab-Tek TC chamber slides (Fig. 2). In a few hours, cell monolayers are formed. After an incubation of 24 hr to allow replication of unneutralized virus, the monolayers are fixed with acetone, stained with fluorescein-conjugated anti-rabies globulin, and examined by UV microscopy. The principle, illustrated in Fig. 3, is that rabies-specific antibodies present in the test serum samples will neutralize the challenge virus, thereby preventing infection of the BHK-21 cell monolayers. In the absence of replicating viral antigen, specific fluorescence will not be observed. Conversely, the presence of specific fluorescence indicates viral replication and therefore absence of neutralizing antibody.

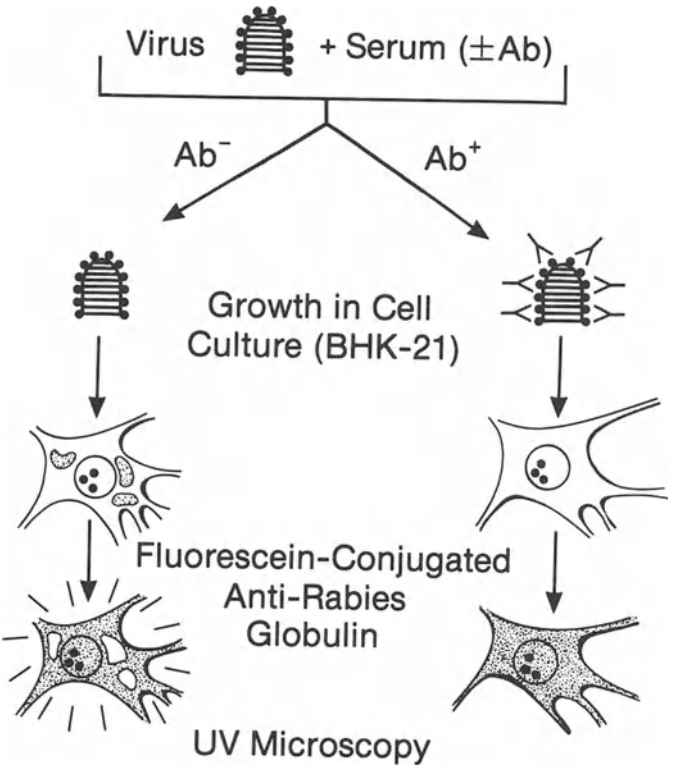

Figure 3. Schematic outline of an immunofluorescence-based neutralization test such as the RFFIT or FIMT. See text for further details.

A microscale modification of the technique, the fluorescence inhibition microtest (FIMT), has been described by Zalan *et al.* (23). This utilizes 96-well polystyrene microtitration plates in place of the 8-chamber Lab-Tek chamber slides of the RFFIT (Fig. 2), thereby facilitating the handling of large numbers of specimens.

Results with the RFFIT (and FIMT) have been shown generally to give close correlation with the MNT in measuring neutralizing antibodies (4,24-27) and this, coupled with the fact that the former is relatively inexpensive and can be completed within 2 working days, justifies its widespread acceptance in many laboratories. However, two recent reports (28,29) have claimed that titers of rabies immune

globulin (RIG) obtained by MNT may be up to 10-fold higher than by RFFIT. This being the case, use of the RFFIT as the assay would result in RIG preparations containing up to 10 times the usually recommended antibody concentration, administration of which could interfere with active antibody formation in post-exposure vaccinees (28-30).

Drawbacks of the RFFIT and FIMT include their requirement for highly specialized equipment and laboratory facilities for handling cell cultures and live rabies virus. Additionally, since they function by measuring the ability of specific antibody to inhibit challenge viral growth in cell cultures, the presence in a serum sample of any non-specific (i.e., non-antibody) factors that interfere with cell or viral growth may result in false positive reactions. Such non-specific factors, causing cytotoxicity, have been encountered more frequently in sera from some animal species (e.g., skunk (*Mephitis mephitis*) and raccoon (*Procyon lotor*)) than others (e.g., human, fox (*Vulpes vulpes*), and domestic animals) (31).

The Soluble Antigen Fluorescent Antibody Test

Although the RFFIT and IFRA tests are sensitive and specific, satisfactory performance requires a high quality UV microscope and a skilled operator to differentiate between specific and non-specific fluorescence. The soluble antigen fluorescent antibody (SAFA) test circumvents the subjective and sometimes tedious aspects of their performance by eliminating the need for microscopic evaluation (32). Instead, purified viral antigen, adsorbed to cellulose acetate discs, is incubated first with test serum dilutions and then FITC-conjugated anti-species antibody. Bound fluorescence is then measured in a fluorometer with the intensity of fluorescence, relative to antibody-negative controls, providing a measure of the rabies antibody titer.

Technically much simpler than the RFFIT, the SAFA test has the disadvantage of requiring high concentrations of purified antigen, purification being essential to reduce non-specific fluorescence to an acceptable level. Manipulation of single paper discs also makes the assay somewhat cumbersome for routine use. Although it is a primary binding assay, as is the IFRA, use of purified antigen as substrate reduces the possibility of non-specific reactions.

THE MIXED HEMADSORPTION TEST

The mixed hemadsorption (MH) test, originally described for rabies by Espmark *et al.* (33), utilizes cell monolayers confluently infected with rabies virus and overlaid with semi-solid nutrient agar. Filter paper disks soaked in undiluted test serum are placed on top of the overlay. During a subsequent 48 hr incubation, antibodies from the serum diffuse through the agar layer to the cell monolayer, and react with viral antigen expressed on the surface of the infected cells. Following removal of the agar layer, presence of rabies-specific antibody is demonstrated using sheep erythrocytes coated with anti-species antibody. The diameter of the zone of hemadsorption was found to be linearly related to the logarithm of the serum dilution or antibody concentration (18). The MH test has been demonstrated to show close correlation with the mouse neutralization test, although some discrepancies, thought to be associated with high IgM antibody levels, have been observed (18).

THE COMPLEMENT FIXATION TEST

The complement fixation (CF) test measures the presence of antibodies that "fix" complement, inhibiting the latter from reacting with an amboceptor (hemolysin), and thereby inhibiting hemolysis of sheep erythrocytes (the indicator system) (for reviews see refs. 34,35). Successful application of the test for rabies requires highly standardized sheep erythrocytes, hemolysin and complement, as well as pure concentrated antigen (36). Even so, CF antibody results have not been found to correlate well with MNT or RFFIT results (37), and the assay has not found widespread use.

The Immunoadherence Hemagglutination Test

A variant of the standard CF test, the immunoadherence hemagglutination (IAHA) test is based on the fixation of complement by complexes of viral antigen with specific antibody, with unreacted complement being measured by agglutination of human erythrocytes bearing receptors for the C3 component (38). The extent of agglutination, therefore, is inversely related to the level of specific CF antibodies in the test sample. Budzko and coworkers (39) found close correlation between results of this test and the RFFIT, although the latter appeared to be

more sensitive in detecting low levels of rabies antibody. The IAHA is simpler to perform than the CF test, and has been reported to be more sensitive, and more sparing of viral antigen (38,39).

HEMAGGLUTINATION-BASED TESTS

These tests, reviewed elsewhere (40-42), rely on the principle that certain biological materials, including many viruses, have receptors that can attach to erythrocytes, thereby causing readily-identifiable hemagglutination (HA) reactions. Tests that fall into this category are rapid, inexpensive, simple to perform, and require little in the way of specialized equipment.

The Direct Hemagglutination-inhibition Test

The interaction of rabies virus and erythrocytes involves electrostatic binding between surface receptors on both virus and cells. Virus-specific antibodies that bind to, or mask, the virus receptor sites will inhibit the HA reaction (for reviews see 40,41). Measurement of the hemagglutination-inhibition (HI) activity of a serum sample, therefore, will provide a measure of its specific neutralizing antibody titer.

Hemagglutination by rabies virus was first described by Halonen *et al.* (43) and shortly thereafter by Kuwert *et al.* (44). Optimal conditions, which include low temperature and a pH of 6.2, are quite similar to those established for rubella and other togaviruses (43). As with the togaviruses, rabies HA is extremely sensitive to inhibition by nonspecific factors in serum, particularly serum lipoproteins, and removal of these inhibitors without a concurrent decrease in antibody titer has been found to be very difficult (45). This problem, and a lack of sensitivity, has prevented wide-spread acceptance of the assay. However, Chappuis and Tixier (46) reported some technical improvements of the earlier protocols, including pre-treatment of the indicator goose erythrocytes with trypsin, and found good correlation of results obtained with the HI test, RFFIT and MNT. Mifune and coworkers (47) have also described an improved HI test for rabies antibody utilizing colloidal silicic acid for removal of nonspecific inhibitors, and bromelain-treated goose erythrocytes for increased hemagglutinating capacity.

The Passive Hemagglutination Test

Whether or not they actively hemagglutinate, viruses can still be used in HA reactions by chemical coupling to erythrocytes with tannic acid or chromium chloride. Cells sensitized in this way will not interact, but will agglutinate in the presence of virus-specific antibody (for reviews see refs. 41,42). This procedure is termed passive, or indirect, hemagglutination, and has been applied to the determination of rabies antibodies by Dierks and Gough (48,49). The test has been found to correlate well with the MNT for samples obtained late in the immune response, when antibody is primarily IgG, but early IgM antibody produced a falsely elevated titer since this is generally more efficient in agglutination reactions.

RADIOIMMUNOASSAY

The basic principle of radioimmunoassay (RIA) techniques is similar to the fluorescence-based assays described earlier: tagged antibodies are used to measure the level of specific antibody-antigen interactions. In RIA techniques, however, the tag is a radioactive isotope such as ^{125}I-iodide, which can be readily incorporated into the phenolic groups of tyrosine residues in proteins (see, e.g., ref. 50). There are many different ways of applying RIA techniques (for reviews see refs. 50-52). The liquid phase procedure for detection of rabies-specific antibodies described by Wiktor (53) involves the separation of labelled antibody-antigen complexes from free (unlabelled) antigen by precipitation with anti-immunoglobulin raised in a different species. The level of radioactivity in the precipitate is directly proportional to the amount of antibody present in the original sample. This test was found to be more sensitive than the MNT, particularly for the detection of early antibody, but the liquid phase system is somewhat cumbersome to use. Another RIA has been reported that used rabies antigen adsorbed to a solid phase (polyvinyl chloride microplate), making the assay more convenient for large scale testing (54). Rabies-specific antibody bound to the antigen was measured using ^{125}I-labelled anti-species antibody.

RIA techniques have been successfully applied to the serology of many viral diseases and they have a number of advantages: they are

rapid, sensitive, and simple to perform. Among their disadvantages are the potential hazards inherent in use of radioisotopes, and the need for costly radioactivity counters. Any potential hazards are mainly associated with the initial preparation of the radiolabelled reagents, however: the amount of radioactivity involved in the individual assays is extremely low. Disposal of radioactive waste may be a problem for some large users, although the fairly short half lives of commonly used isotopes such as ^{125}I (2 months) facilitate this process. These same short half lives, however, mean that the shelf life of labelled reagents is limited to a few months, necessitating repeated preparations and standardizations.

With regard to the correlation of results obtained with RIA procedures and other tests such as the RFFIT and MNT, much depends on the state of the reagents used. Since it is a primary antibody-binding assay rather than a neutralization one, the degree of purity of the viral antigen, for example, will affect the specificity of antibodies measured (neutralizing, non-neutralizing, etc.). This is discussed further in the following section.

ENZYME-LINKED IMMUNOASSAY

In the 17 years since it was first described by Engvall and Perlmann (55), the enzyme-linked immunosorbent assay (ELISA or EIA) has become one of the single most widely used virological diagnostic technique (for reviews see refs. 56-63). While identical to the RIA in principle, it offers a number of practical advantages, including the use of stable, non-hazardous reagents, without the requirement for costly monitoring equipment.

Many different forms of ELISA systems exist, but most reported antibody assays for rabies follow an indirect protocol (64-70). One version, illustrated in Fig. 4, is carried out as follows:

(i) Viral antigen (whole virus or purified G protein) is adsorbed to a solid phase such as the wells of a 96-well microtitration plate.

(ii) Test sera are added, and incubated to allow any rabies-specific antibody present to react with the fixed antigen. Unreacted serum components are then removed by washing.

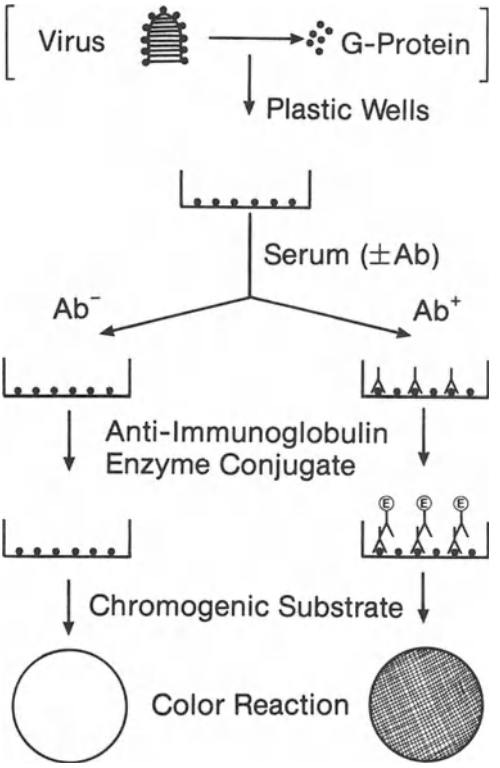

Figure 4. Schematic outline of an indirect ELISA reaction for rabies-specific antibodies. See text for further details.

(iii) An anti-species globulin conjugated with an enzyme such as horse-radish peroxidase or alkaline phosphatase is added, which reacts with antibody bound in step 2. Again, unreacted conjugate is removed by washing.

(iv) Level of bound conjugate, which is proportional to the amount of bound rabies antibody, is then measured visually or photometrically following reaction of the enzyme with a chromogenic substrate system.

If specimens of more than one species are to be assayed, the above protocol may require the preparation of anti-globulin/enzyme conjugates for each one. In this case, it may be more convenient to substitute an unconjugated anti-globulin in step (iii), and to add an extra step in

which an enzyme-labelled anti-globulin directed against the first anti-globulin is added. This double antibody technique permits the use of more readily available commercial immunological reagents. It should be noted, however, that antibodies are not necessarily monospecies-specific. For example, anti-canine immunoglobulins react strongly with the corresponding globulins of a number of other carnivores. Enzyme-conjugated anti-canine globulins are readily available commercially, and have been used to assay antibodies in sera of the red fox, striped skunk, and raccoon, as well as of domestic cats and dogs (71).

With some further modification of these protocols, the ELISA permits measurement of class-specific immunoglobulins, e.g., IgM (31,64,68,70). In this case, enzyme-labelled anti-IgM (heavy chain specific) is added instead of labelled anti-whole immunoglobulin (64,68,70), or unlabelled anti-IgM followed by labelled antibody against this second antibody (31). There are certain problems associated with this type of IgM assay, however, including potential interference with rheumatoid factor, and competition between IgG and IgM for antigen binding sites. These, and ways of dealing with them have been analysed elsewhere (61,72).

An alternative ELISA protocol uses enzyme-labelled staphylococcal protein A in place of the anti-species antibody (65,69); however, this cannot be used for identification of class-specific immunoglobulins. Protein A binds to the Fc portion of immunoglobulins of a number of species (73). In human serum, the binding is principally to subclasses 1, 2 and 4 of IgG although some IgM and IgA reactivities have also been reported (74,75). In canine serum, protein A is capable of binding >99% of the IgG and up to 90% of the IgM antibodies (76). This may be an advantage or disadvantage, depending on the aim of the particular assay.

An innate characteristic of the ELISA is that it measures antibody binding and not neutralization. As with all antibody binding assays, results may be influenced by the avidity and affinity of antibodies as well as their concentration (78), antigen density (78,79), and even the epitope density of the antigen used (80). The nature of the antigen used may also affect the results obtained. Neural tissue vaccines, for instance, stimulate production of a high level of non-

neutralizing anti-nucleocapsid antibodies. If whole virus is used as immunosorbent in an ELISA to test the effectiveness of such vaccines, a titer may be obtained that is not indicative of the level of protection (33). Procedures using purified G protein (31,65,71) instead of whole virus will provide results that correlate more closely with neutralization tests. Even so, it must be noted that there are some epitopes present on the G protein that induce production of non-neutralizing antibody (82,83).

There exist several different methods for expressing ELISA results, although in rabies serology the end point titration method (64,70,84-86) and the single dilution method using standard dilution curves (31,67-69,71) are the most common. The single dilution method, however, is the more practical for testing large numbers of samples.

A test has recently been developed which incorporates features of both the FIMT and the ELISA (87). The procedure follows the initial steps of the FIMT: incubation of virus and test serum dilutions in microtitration plates, followed by addition of BHK-21 cells and incubation for a further 24 hrs. Instead of reaction with fluorescein-conjugated anti-rabies globulin, however, the cells are disrupted by freeze-thawing and liberated virus is measured enzymatically by an ELISA protocol. By this technique the ELISA is actually a measure of neutralization, although it is technically more demanding, and suffers from some of the problems inherent in the tissue culture-based test. As with the FIMT and RFFIT, any non-specific factors affecting cell or viral growth may affect the results of this test.

DISCUSSION

From the number of rabies antibody tests currently available, it is apparent that no single test is clearly superior in all respects to the others. In individual laboratories, selection of assay procedures must depend on a number of factors, including the volume of samples to be processed, the financial budget and technical facilities available. The personal preferences and level of expertise of the investigators are also considerations.

To illustrate the above, the MNT is technically fairly undemanding and needs no specialized laboratory equipment other than that required

for the housing of rabies-infected animals, but a large budget would be necessary for use of this test on a large scale. The widely used alternative, the RFFIT, dispenses with the need for animals, and unit cost per test is only a small fraction of that by the MNT. However, it substitutes requirements for a high level of technical expertise, and the need for cell culture facilities and a high quality UV microscope. For these reasons, the RFFIT is best suited as a routine test procedure for the specialist laboratory. Even in such laboratories, however, the ELISA, by virtue of its simplicity, rapidity and reproducibility, is being increasingly used as a screening method, with the use of fluorescence-based assays being reserved for confirmation of results.

Some assays require only basic laboratory equipment. These include the CF, IAHA, HI and passive HA tests, which have the additional biosafety advantage of not requiring live virus. In each of these, titration endpoints are determined simply by visual inspection of erythrocyte patterns: agglutinated, non-agglutinated, or hemolysed. The ELISA can also fall into this category since visual estimation of the intensity of the color reactions, relative to positive and negative controls, can provide a qualitative measure of antibody levels (although specifically designed microplate readers are commercially available for quantitative measurement). Lack of facilities for production of viral antigen may limit the usefulness of even these tests for some laboratories; however, it should be noted that the ELISA can be carried out using commercial rabies vaccine as antigen (68,84). The availability of a commercial ELISA kit for determination of rabies antibodies (EIA-RAGE; Institut Pasteur Production, Marnes la Coquette, France) further facilitates use of this assay.

In conclusion, then, there are presently available a number of acceptable rabies antibody tests to fill the requirements of different laboratory facilities. Some are based on virus neutralization reactions, and therefore specifically measure neutralizing (protective) antibodies. Others, usually simpler to perform, measure antibody binding reactions, not all of which may be virus neutralizing. Careful attention to the purity of the antigen and immunological reagents used, however, may permit selective measurement of specific antibody types.

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11

EPIZOOTIOLOGY OF RABIES: EURASIA AND AFRICA

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INTRODUCTION

It is a formidable task to summarize, in a fairly short chapter, the epizootiology of rabies in Eurasia and Africa. Indeed, these 3 continents presently contain almost all types of rabies, some of which are in a constant state of evolution. To simplify matters, we have adopted the following plan of exposition for each continent: a brief history of rabies; general characteristics of the current epizootiological situations (viruses, vectors); official statistics of the disease in each country; effect of preventive measures; and general trends in epidemiological evolution. First of all, however, we shall review some basic information regarding vectors, viral strains, and statistical evaluations.

BACKGROUND

The epidemiological state of a continent, or of the various regions that constitute it, is generally characterized by 2 types of data: *quantitative* (statistics of the number of cases of rabies or, in default of these, the number of human anti-rabies treatments) and *qualitative* (nature of the animal vectors and the rabies viral isolates).

1. The Statistical Data

Cases of Animal and Human Rabies

With rare exceptions, the figures reported in this chapter are those officially available from national agencies, and relate only to cases of laboratory-confirmed rabies. Laboratory diagnoses considered

acceptable are carried out by the specific immunofluorescence technique, by mouse inoculation, or by examination for Negri bodies (see Webster and Casey, this volume). It is clear that the published laboratory figures reflect only a small proportion of the true cases of rabies. Reliance on clinical diagnostic data is not highly reliable, therefore, although the figures may provide an indication (and perhaps the only one) of the incidence of rabies in a particular country.

Number of Human Post-exposure Treatments

In certain countries without diagnostic centers, but in which rabies is prevalent, the only available statistics are those of the number of human post-exposure treatments. From relationships established in better-equipped neighboring countries with comparable epidemiological situations, however, it is possible to obtain an approximate idea of the incidence of canine rabies. Comparative studies of the statistics of certain countries of Africa and the Orient have established the following general relationship: for every 2,500 human treatments there is a yearly average of 4 cases of human rabies and 60 canine cases, corresponding to a canine population of 100,000 animals and a human population of 1,000,000 (see ref. 6).

2. The Viruses and Vectors of Rabies

The Viruses

According to the World Health Organization Expert Committee on Rabies (1), there are presently 4 serotypes of rabies virus (*Lyssavirus*, Rhabdoviridae) that can be distinguished on the basis of cross-protection tests in mice. Serotype 1 (prototype: Challenge Virus Standard, or CVS) is considered to be the only "true" rabies virus. The 3 others are the "rabies-related" viruses: serotype 2 (prototype: Lagos bat), serotype 3 (prototype: Mokola) and serotype 4 (prototype: Duvenhage). Use of monoclonal antibodies (1-4) has permitted further differentiation of these serotypes into numerous variants or isolates that are frequently characteristic of a particular geographical region (see King and Crick, this volume).

In certain specific cases, notably in Africa, the rabies-like viruses have their own epizootiological roles (2). Some isolates have been obtained from within serotype 1 that appear to have acquired

a particular tropism for one or another animal species, which has permitted them to be categorized as vulpine, canine, etc., "biotypes" (5). All these serotypes, isolates or biotypes can play different roles depending on their geographical location, and may create complex epidemiological situations. It should be kept in mind, however, that most human fatalities worldwide (around 50,000 per year) have been reported following exposure to rabies virus of *canine* origin (3,4).

The Vectors and Reservoirs

In rabies terminology, the term "*vector*" refers to a species of animal mainly responsible for maintaining an epizootiological cycle in a particular geographic region at a particular time. Without this (or these) species, rabies would disappear spontaneously, since other animal species, including man, are simply victims of "spill-over" from the principal cycle. The situation is more complicated when 2 or more vectors co-exist, but such a co-existence is relatively rare, and seems to be largely excluded even when there are repeated contacts between competing vectors. In these situations, the (heterologous) virus of 1 of the vectors may act as a "live vaccine" for the other vector(s) (5).

Contrary to some other diseases, rabies does not seem to reside in "reservoir" species that harbor the virus without symptoms (e.g., rodents, or other vertebrates or invertebrates). However, animals of the vector species may harbor the virus for very long periods of time during prolonged incubation periods. This provides a means of survival of the virus between epizootic outbreaks.

RABIES IN EURASIA

1. Historical Notes

Eurasia is the continent for which we have the oldest information on the nature and distribution of rabies, dating back to the 23rd century B.C. in Mesopotamia. In the centuries that followed, references to human and canine rabies have been made repeatedly in the codes, narratives, poems, paintings, sculptures, mosaics, etc., of Asiatic, Oriental and European civilizations (7). One can therefore consider Eurasia as a seat of permanently endemic rabies, with the exception of certain areas (e.g., islands) and certain times.

Some dates can provide historical landmarks, or can demarcate important stages in our knowledge of the epidemiology of rabies. There are, for example, the following:

- 782-500 B.C.: first mention, in China, of the destruction of rabid stray dogs, and cauterization of wounds.

- Aeschylus (525-456 B.C.) is immersed in the sea as treatment for the bite of a rabid dog.

- Aristotle (384-322 B.C.) describes cases of rabies in horses and camels.

- 1st century A.D.: Celsus writes "*Autem omnis morsus habet fere quoddam virus*", indicating that the "poison" (virus) is contained in the saliva of rabid dogs.

- 900 A.D.: first important mention of sylvatic rabies. A rabid bear mortally infects 20 inhabitants of Lyon (France).

- 11th century: Avicenna gives a complete description of the general symptoms and local erythema observed following a rabid bite.

- 1272: first report of an important episode of wolf rabies, in Franconia (many domestic animal deaths, 30 human deaths).

- 1500: important epizootic of canine rabies in Spain, then over the entire continental Europe (1586) and England (1734).

- 1803: Zincke demonstrates experimentally the virulence of canine saliva. Several hundred rabid foxes reported in the Jura region (1803-1840).

- 1879: Galtier transmits rabies to rabbits, and vaccinates sheep by injection.

- 1885: Pasteur demonstrates the possibility of combating canine rabies by vaccinating dogs (before infection) and man (after infection) with progressively desiccated preparations of rabies-infected spinal cord.

- 1945: Progressive spread of vulpine rabies in Europe.

2. General Characteristics of the Current Epidemics

As in Africa, Eurasian rabies can be defined by a number of general characteristics applicable to the whole continental mass. These characteristics relate essentially to the types of rabies (canine or sylvatic), the types of virus, and the different vectors of these viruses.

2.1. The Types of Rabies in Eurasia

The currently prevalent type of rabies in most of continental Europe is vulpine (sylvatic), whereas the dominant type in Asia is canine. The principal victims in Europe and Asia, therefore, are respectively the red fox (*Vulpes vulpes*) and the domestic dog (*Canis familiaris*), together representing over 75% of all rabid animals. In their respective continents, these 2 species constitute the reservoirs and vectors of specifically-adapted rabies viruses.

In some countries (e.g., some republics of the U.S.S.R. and Yugoslavia), the 2 types of rabies co-exist (Fig. 1). It should also be noted that, for several decades, a new species of wild carnivore, the raccoon dog (*Nyctereutes procyonoides*) has been a rabies vector in some U.S.S.R. republics and Poland. Additionally, since 1985, an insectivorous bat (*Eptesicus serotinus*) has fallen victim to a new rabies-related strain (serotype 4) in countries bordering the North and Baltic Seas (Denmark, Germany, Netherlands), and, in 1987, Spain.

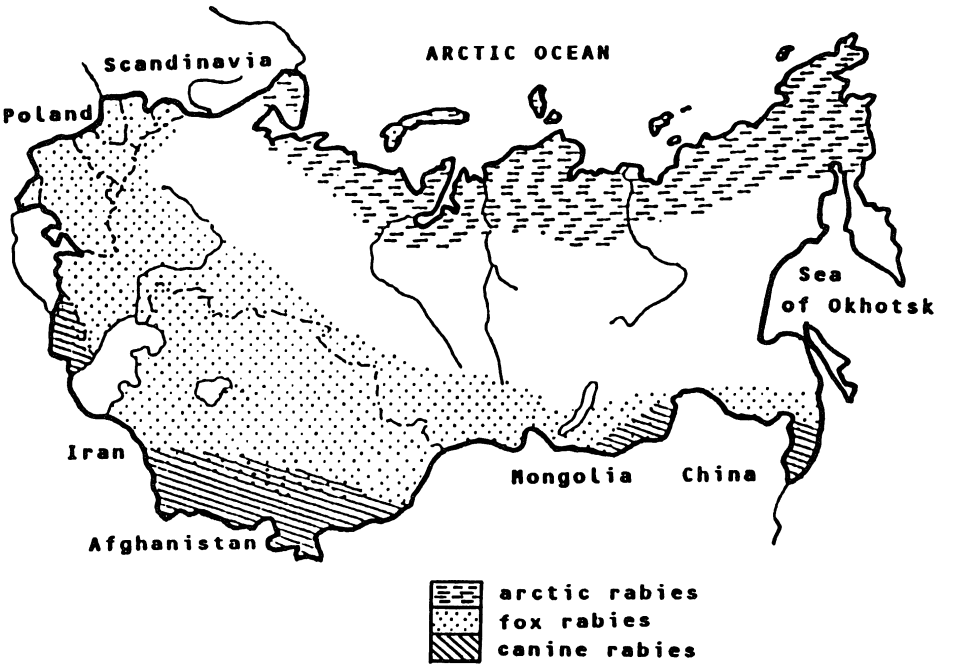

Figure 1. Distribution of arctic, fox, and canine rabies in the U.S.S.R. Modified from ref. 11.

2.2. The Viruses

In contrast to the situation on the African continent, Eurasia appears to harbor only a limited number of rabies strains, most belonging to serotype 1. Since 1985, however, some virus strains have been isolated from European insectivorous bats that closely resemble the known rabies-related viruses (see King and Crick, this volume).

Viruses of Serotype 1

Viruses of this type currently predominate over all Eurasia. Several variants have been identified on the basis of their reactions with monoclonal antibodies, but these do not appear to differ in their pathogenic or immunogenic properties in mice. By contrast, studies of the pathogenic and immunogenic properties of isolates made from within serotype 1 itself, and grown in heterologous hosts (fox, dog, raccoon dog, etc.), have permitted identification of "biotypes" fully adapted to each of these species (5).

Viruses of Other Serotypes

Certain virus strains, isolated from species other than European fox or dog do not appear to belong to serotype 1. Indeed, by their monoclonal antibody patterns, they seem to be closer to the rabies-related viruses. This is particularly the case with the viruses isolated from European bats, which closely resemble serotype 4 (Duvenhage) (8).

2.3. The Vectors

The dog (*Canis familiaris*) is a major vector of rabies over the length of the Asiatic continent, from the Sea of Japan to the Red Sea, and in the southern part of Europe. The division between canine and fox rabies follows approximately the line between Belgrade and Khabarovsk, passing through Baku, Tashkent and Irkutsk (Fig. 1).

The red fox (*Vulpes vulpes*) (Fig. 2) is the dominant vector in central and western Europe north of the line defined above and extending to the limits of the arctic fox (9). The red fox has also recently become an important vector in Israel. The arctic fox (*Alopex lagopus*) is a vector only in the northern U.S.S.R. (10).

Figure 2. Rabid red fox (*Vulpes vulpes*): furious form.

Figure 3. Raccoon dog (*Nyctereutes procyonoides*). This Asiatic species is progressively invading Europe, and is the vehicle for a new biotype of rabies virus adapted to its host and vector.

The raccoon dog (*Nyctereutes procyonoides*) (Fig. 3) is an increasingly important vector of rabies, co-existing with the red fox in some regions of the U.S.S.R. (Byelorussia, the Baltic) and in Poland. Insectivorous bats (*Eptesicus serotinus*) have been identified recently as being the only victims of rabies in Denmark and as occasional victims in northern Germany, the Netherlands, and Spain (8).

Other species, whether domestic (e.g., cats, herbivores, pigs) or wild (e.g., mustelids, wild cats, jackals), are not considered vectors but simply as victims of independent cycles established by dogs, red or arctic foxes, or even fruit-eating bats in certain regions of Asia such as Thailand (4,12). Nevertheless, the wolf (*Lupus lupus*) still plays an important role as vector, along with the dog, in some regions of Iran, Afghanistan, Iraq and the U.S.S.R.

3. Epidemiological Statistics

Table 1 summarizes official rabies data from European and Asiatic countries. The following provides a more detailed commentary on the individual continents.

Europe

European rabies data are of high quality, compiled from generally reliable epidemiological surveys conducted throughout western Europe and coordinated by the W.H.O. Collaborating Center at Tübingen, Federal Republic of Germany. Since 1977, this Center has published the "Rabies Bulletin Europe" which assembles quarterly all data received in each case of human and animal rabies. On the basis of data in the latest issue of this bulletin, the current situation can be summarized as follows:

Rabies-free Countries

These include Great Britain and Ireland, Scandinavian countries (Norway and Sweden), Albania, Bulgaria, the Iberian Peninsula (Spain and Portugal), Greece, and the islands of Cyprus and Malta. However, occasional cases imported from neighboring countries have been reported even in these. Mainland Norway is rabies-free, but some Norwegian islands are contaminated with fox rabies. Bulgaria, although officially rabies-free, conducts an average of 3,727 post-exposure anti-rabies treatments yearly.

Table 1. Epidemiological Statistics of Rabies in Eurasia

Country	Period Covered	Av. Annual Laboratory-confirmed Cases in: Dogs	Wildlife	Humans	Average Annual Human Treatments
Europe:					
Austria	1985/86*	3	1,326	None	1,315
Belgium	1985/86*	9	193	None	853
Czechoslovakia	1985/86*	36	1,392	None	2,769
Denmark	1985/86*	None	105	None	32
France	1985/86*	52	2,046	None	7,477
Germany, Fed. Rep.	1985/86*	45	4,409	None	-
Germany, Dem. Rep.	1985	-§	-	-	4,139
Hungary	1985/86*	46	1,091	None	2,351
Italy	1985/86*	1	28	None	-
Luxembourg	1985/86*	None	92	None	109
Poland	1985/86*	48	883	None	2,803
Rumania	1985/86*	6	37	None	-
Switzerland	1985/86*	1	165	None	-
Turkey	1985/86*	848	46	None	-
U.S.S.R.	1978-83	1,179**			-
Yugoslavia	1985/86*	11	469	None	4,264
Asia:					
Afghanistan	1962-83	-	-	-	823
Bangladesh	1985	16	1	1,950	-
Bhutan	1980	-	-	-	296
Burma	1975-83	-	-	40	2,000
India	1985	2,200	-	25,000	500,000
Indonesia	1976-80	3,872	-	312	-
Iran	1982-83	450	-	18	51,500
Iraq	1985	-	-	3	49,500
Israel	1985	2	10	None	900
Jordan	1985-86	9	2	0.5	550
Laos	1986	85	-	-	-
Lebanon	1966-68	10	-	1	-
Malaysia	1985	2	None	None	-
Nepal	1983	210	-	-	107
Pakistan	1977	8,188	-	503	30,000
Philippines	1982-83	101	-	208	19,000
Sri Lanka	1960-80	852	-	510	75
Syria	1984	-	-	-	2,585
Thailand	1985	7,491	-	206	72,000
Vietnam	1977-79	10,244	-	95	-
Yemen	1985	169	-	-	-

* Rabies cases reported for 1985, human treatments for 1986.
§ Official data not available.
** Total reported cases, without breakdown (57% in Russia and Ukraine).
Note: The following have reported only sporadic cases of rabies: Bahrain, Brunei, Kampuchea, North and South Korea, Kuwait, Macau, Mongolia, Oman, People's Republic of China, Quatar, Saudi Arabia, Singapore, and the United Arab Emirates. Taiwan is rabies-free.

Countries with Canine Rabies

In Turkey, southern U.S.S.R. and southern Yugoslavia, cases of rabies are mainly of canine origin.

Countries with Fox or Bat Rabies

Germany (East and West), Austria, Belgium, Czechoslovakia, France, Hungary, Italy (except in 1987), Luxembourg, the Netherlands, Poland, Rumania, Switzerland (and Liechtenstein), U.S.S.R. and northern Yugoslavia. Fox rabies reached these countries at different times, spreading eastward and westward (from Poland) from 1950-1980 at a rate of 40 km annually (Fig. 4). Denmark is free of rabies in terrestrial animals but since 1985 has been heavily infected with bat rabies (more than 110 cases in 1986).

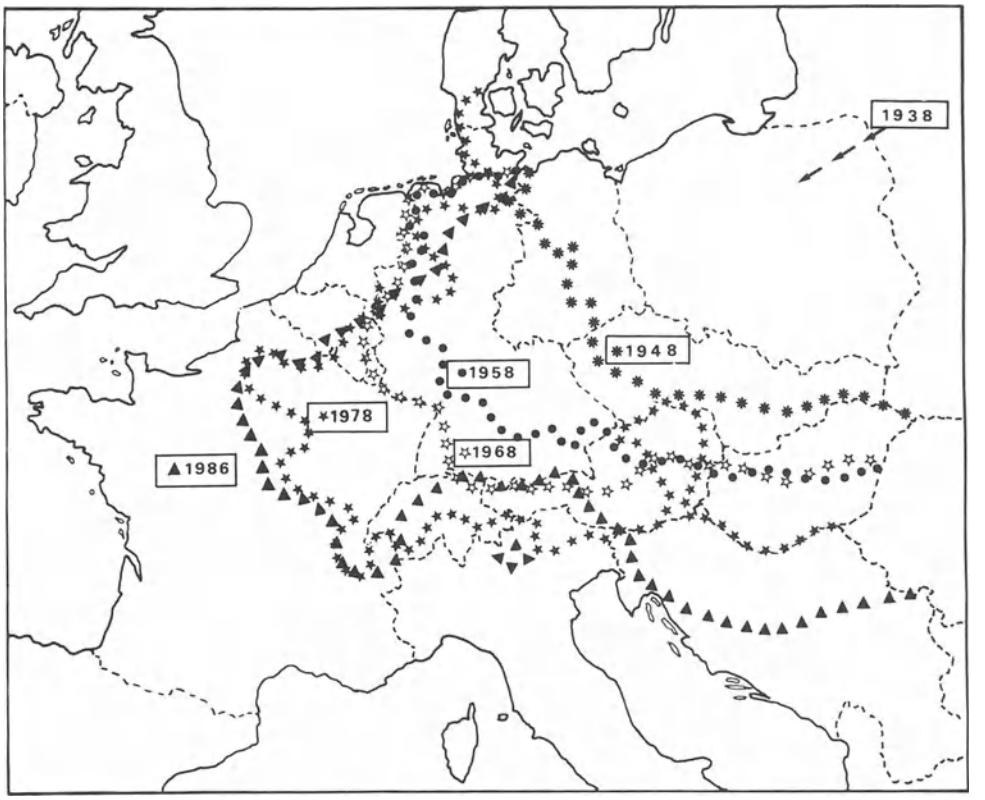

Figure 4. Advance of the rabies front in Europe, 1948-86.

Asia

Rabies data on Asian countries are also summarized in Table 1. The official figures of incidence in humans or animals sometimes vary considerably, but this is more often due to variations in the efficiency of the disease surveys than to real differences in the epidemiological picture. All these countries, no matter which, have 1 point in common: practical experience only of canine rabies (Thailand, heavily infected with canine rabies, has had episodes of cat and rodent rabies, but the independence of these cycles has not been clearly proven (4)). Despite this uniformity, however, some specific characteristics can be noted for certain regions (3):

Rabies-free Regions

These include cities and islands in which it has been possible to eliminate the disease and to prevent its reintroduction: Japan, Taiwan, some small islands in Indonesia and the Philippines, and the peninsulas of Malaysia and Hong Kong. This favorable situation is most often the result of application of canine vaccination (e.g., yearly vaccinations, as in Japan) or of very strict canine control (legislative, or as a result of the animals being used as food in some countries), or the 2 measures conjointly (e.g., as in Taiwan).

Regions with Low Level Rabies

These include those in which the destruction of stray dogs is not prohibited by religions such as Buddhism, and in which control measures are rigorously applied (e.g., Hong Kong, Malaysia, some Chinese provinces, etc.).

Regions with Endemic Rabies, with Annual Fluctuations

All other Asian countries fall into this category. The incidence of disease appears all the more spectactular because of the vast land mass involved. For example, a large percentage of worldwide human mortalities occurs in India alone (3,4).

4. Effect of Prophylactic Measures - Current Trends

There is considerable variation in the current status, depending on the continent, Europe or Asia, or even in different countries within these continents.

Europe

In general, countries which have had canine rabies have been able to rid themselves of it by rigorous application of medical prophylactic (vaccination) or sanitary measures (stray dog control). With the appearance of vulpine rabies the situation has not been as easy to control, since it is much more difficult to reach the reservoir by the above 2 techniques. Fox population control has resulted in a reduction in the number of infections of domestic animals and humans, but with only a few exceptions (e.g., Denmark), such measures have not succeeded in eradicating rabies (1,4,13). In contrast, oral vaccination of foxes has had more definitive results, particularly in Switzerland (1978-), W. Germany (1983-) and recently Austria, Belgium, France, Luxembourg, and Italy (1986-). Simultaneous application of both approaches, as currently implemented in Europe, permits optimism that western Europe will increasingly be freed from rabies (see Wandeler, this volume).

The situation is less favorable in Central Europe and the U.S.S.R, regions in which vaccination of foxes has not yet been undertaken on a large scale (12).

Asia

Depending on the country, measures taken to combat canine rabies (the only form present) are extremely varied in their application and results (4). The control of stray dogs is rarely effective in a rapidly expanding human and canine demography, and the attraction which urban and peri-urban areas exert on these populations renders access difficult to teams responsible for control. Only some countries or cities, well endowed with personnel and equipment (e.g., Japan, Taiwan, Hong Kong, Malay peninsula) have succeeded in this endeavor. Vaccination of dogs is effective only if 75% of the population is reached. This is very rarely the case, except in major cities, despite remarkable efforts to develop programs meeting the criteria of the W.H.O. (e.g., Nepal, Sri Lanka, the Indonesian islands).

Overall, then, it can be concluded that, with a few very specific exceptions, the canine rabies problem in Asia is not on the road to improvement but will remain unchanged or will even spread in the years to come. The political instability of several regions of Asia (e.g., Iraq, Iran, Sri Lanka, Yemen, etc.) will undoubtedly favor this trend.

RABIES IN AFRICA

1. Historical Notes

The African continent has probably been infected with rabies since ancient times. Its continuity with the Middle East, where written accounts of the disease date from the earliest antiquities (7) permit the conjecture that its northern regions, particularly upper Egypt, were infected during the same time period. Nevertheless, the rarity or even total absence of written records in the majority of its countries does not permit any certainty as to the extent and distribution of cases of rabies before European colonization. Colonization, of course, often coincided with the introduction of laboratory diagnoses, and records, of the disease.

The few reports that provide the most definitive points of reference are those obtained from the following regions:

- 1780: a case of rabies is recorded in South Africa by Thunberg (see ref. 22).
- 1858: an episode of canine rabies in Algeria requiring specific prophylactic measures.
- 1862: while exploring the sources of the Nile, Sir Samuel Baker notes that rabies is epidemic in the whole of Abyssinia (Ethiopia).
- 1881: first official report of rabies in Madagascar by J. Pearse (*"Medical Mission Work in Madagascar"*) but oral tradition makes reference to much earlier episodes of the disease.
- 1887: rabies epidemic in cattle in South West Africa (Namibia).
- Early 20th century: in several other African countries (Kenya (1900), Sudan (1904), Mozambique and Nigeria (1912), etc.), rabies is officially confirmed as laboratories are opened.

2. General Characteristics of Current Epidemics

African rabies can be defined by a certain number of general characteristics that apply to the entire continent (including the island of Madagascar which, with human and animal populations of both Asian and African origin, is traditionally associated with the African continent). These characteristics relate essentially to the type of rabies (canine or sylvatic), the types of virus, and the different vectors of these viruses.

2.1. The Types of Rabies in Africa and Madagascar

The type of rabies currently prevalent over the majority of the African continent is unquestionably canine. This is characterized by a perennial enzootic with the domestic dog (*Canis familiaris*) as principal victim, vector, and general reservoir.

Principal victim

The dog has always represented, in most African countries, more than 75% of known rabid animals (14).

Vector

The dog is the only vector of rabies in Madagascar, in North Africa and north of the River Zambezi in Black Africa. Proof of this has been demonstrated by the disappearance of the disease (animal or human) in all countries in which canine rabies has been controlled over a known time period (Zimbabwe, and some northern and southern regions).

Reservoir

To date, no other animal has been identified as being capable of assuring the resurgence of the disease in territories in which only canine rabies is rife. It is probable that, as in the case of other vectors, this would require hosts with incubation periods which conserve the virus for very long periods of time.

Depending on whether canine rabies is endemic in the big urban centers or in rural areas, it may present somewhat different characteristics. Factors such as the size and intensity of infectious foci, the rapidity of spread, difficulty of eradication, etc., will influence these, but the epizootiological mode of transmission remains fundamentally the same in both cases, and exchanges are frequent between urban and rural rabies.

Southern Africa represents a particular case in the dark continent: it is the only part of the continent supporting the existence of cycles of sylvatic rabies that are clearly independent of the canine cycle. These involve certain species of viverrids (yellow mongoose) or herbivores such as kudu antelopes (3) (Fig. 5).

2.2. The Viruses

Rabies-related viruses (serotypes 2,3,4) were first isolated on the African continent, and it is here also that the greatest variations

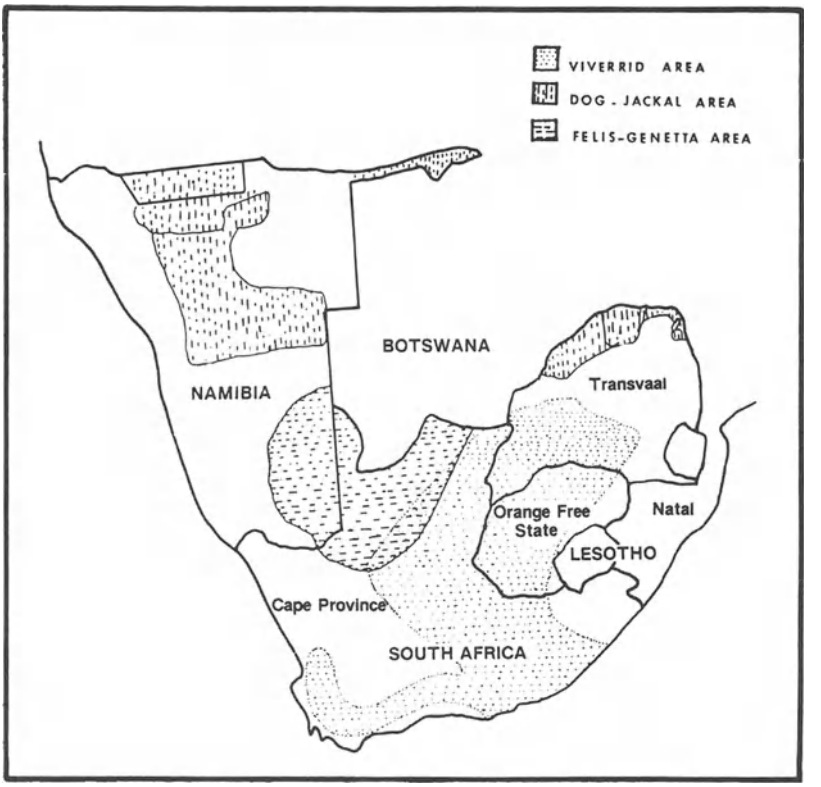

Figure 5. Map of southern Africa showing the different rabies areas in South Africa and Namibia. Figure modified from ref. 20.

in pathogenic potential of true rabies isolates (serotype 1) have been reported.

Viruses of Serotype 1

Until the discovery and application of monoclonal antibodies, all strains of virus of this serotype were believed to be identical, even with strains from other continents. Even so, a strain of canine virus isolated in 1922 in west Africa had long been considered different from the classical strains by having a lower virulence, and was called "oulou fato" ("mad dog disease"), although the validity of this difference was questioned by Remlinger (15), and has not since been convincingly confirmed. In 1957, however, viruses were isolated in Ethiopia (16) which were also considered, and subsequently confirmed (17,18), to have pathogenic properties different from usual strains.

Viruses of Serotypes 2,3,4

It is interesting to recall the history of the 3 strains considered as prototypes 2, 3 and 4. Lagos bat strain (prototype 2) was first isolated in 1956 from the brain of a Nigerian fruit-eating bat (*Eidolum helvum*) and was isolated later from other bats in south and central Africa (1,4,6,19). Strain Mokola (prototype 3) was isolated first in 1968 from a mixture of Nigerian shrew viscera. Named Ibadan shrew virus No. 27377 prior to 1973, it has since been isolated from humans and domestic and wild animals in several African countries. Strain Duvenhage (prototype 4) was initially a human South African isolate (1971) and was later identified in bats from the same region.

Viruses Isolated from Insects

Other rabies-related viruses have been isolated in Africa from insects: strain Obodhiang from *Mansonia uniformis* in Sudan (1963), and strain Kotonkan from *Culicoides* species in Nigeria.

2.3. The Vectors

The dog (*Canis familiaris*) is the major rabies vector over the entire African continent, although in some regions a sylvatic cycle complements it or replaces it (see below). Madagascar, where rabies has existed for centuries, perfectly illustrates this exclusive role of the dog as victim, vector and reservoir of the virus. Indeed, no other wild indigenous species (carnivorous or other) exists on this island that has been found rabid, save for exceptional cases due to canine contamination (3).

The yellow mongoose (*Cynictis penicillata*) is the principal wildlife vector on the central plateau of South Africa (20,21) and is the origin of numerous human cases. It can also infect suricates (*Suricata suricata*) and ground squirrels (*Xerus inauris*) which utilize its burrows. The black-backed jackal (*Canis mesomelas*) plays the role of an additional vector in northern Transvaal and Namibia, in southern Zambia and in some regions of Zimbabwe (3,22). Wild cats (*Felis* sp.) and the genet (*Genetta* sp.) are both considered to be "supplementary vectors" in southern Namibia and the northern Cape (23). Although these wild carnivores can maintain independent rabies cycles, it has not been demonstrated clearly whether the disease is of canine origin or whether they contract it by contact with the same species. Whatever

Table 2. Epidemiological Statistics of Rabies in Africa

Country	Period Covered	Av. Annual Laboratory-confirmed Cases in:			Average Annual Human Treatments
		Dogs	Wildlife	Humans	
Algeria	1980	302	9	69	37,714
Angola	1953-68	62	1	0.1	-§
Benin	1985	160	None	1	-
Botswana	1972-82	29.6	6.5	3.5	169
Cameroon	1964-67	37	0.2	2.2	-
Chad + Central African Republic	1964-68	46	0.8	10.2	-
Congo	1964-87	43	0.3	1.8	-
Egypt	1964-66	47	None	28.2	25,000
Ethiopia	1980-82	112	12	53	2,433
Ghana	1970-82	115.2	0.7	19.7	1,750
Ivory Coast	1981	200	1	-	-
Kenya	1958-82	15.1	3.7	3.5	-
Madagascar	1959-82	47.8	None	0.8	1,317
Malawi	1964-68	129	None	3.8	-
Mali	1967-79	21	None	4	-
Morocco + W. Sahara	1977-84	408	-	32	12,365
Mozambique	1978-82	27	0.5	25	3,450
Namibia	1976-82	20.5	21*	0.7	-
Niger	1964-74	25	0.1	8	-
Nigeria	1969-78	43.5	0.1	16.9	-
Rwanda Burundi	1954-80	40.3	0.6	7.9	520
Senegal + Gambia	1986	75	1	7	1,080
Sierra Leone	1966-68	7	None	3.3	-
Somalia + Djibouti	1981	2	-	-	-
South Africa	1985	125	159	-	-
Sudan	1981-82	131	7	9	11,886
Tanzania	1966-74	52	2.4	8.2	1,050
Togo	1985-86	58	1	8	-
Tunisia	1985-86	38	1	3	15,850
Uganda	1965-66	39	2	11	-
Zaire	1964-67	145	1	-	-
Zambia	1972-82	150.4	25	19.3	2,650
Zimbabwe	1977-79	196	139	18	2,121

* Not including kudu antelopes (38 cases/ year during the outbreak).

§ Official data not available.

Notes: Periods have been chosen as being most representative of the rabies situation. Data presented have been obtained either from official sources (e.g. ref. 25) or from personal communications to the author.

The islands of Cape Verde, Comoros, Mauritius, Réunion, and Seychelles report only sporadic cases of canine rabies, as do the following countries: Burkina Faso, Gabon, The Guineas, Liberia, and Mauritania. Lybia is officially rabies-free. The statistics for Lesotho and Swaziland have been included with those of South Africa.

the case, it is often the dog (infected more easily by canine strains than by sylvatic strains) that man has most to fear (24).

The kudu antelope (*Tragelaphus strepsiceros*) established an independent cycle of rabies in Namibia from 1977 until 1982. Following initial transmission from jackals, more than 50,000 antelopes infected each other lethally, perhaps by commensalism or simple mucosal contact, or by transmission via saliva-coated thorn trees. Since 1983, the epizootic has spontaneously regressed (3). Other species, whether domestic (e.g., cats, herbivores, pigs) or wild (e.g., carnivores, monkeys, insectivores), are not considered to be vectors but simply victims of the independent cycles described previously.

3. Epidemiological Statistics

Table 2, which summarizes official data on rabies in the African countries, must be supplemented by some more detailed comments on each of the large climatic or geophysical regions.

North Africa

Algeria, Morocco, Tunisia, and Egypt have been canine rabies-endemic areas for centuries. The number of recognized cases in dogs exceeds several hundred yearly, and the number of yearly human deaths is of the order of 0.4 per 100,000 inhabitants. Regular control programs have been implemented since 1980. The Tunisian program has resulted in the complete absence of human cases in 1985. Libya, sparsely populated except for the coastal region, has been declared rabies-free.

Sahelian and Sudanese Africa

All countries situated between the Sahara desert and the equatorial zone are uniformly infected with canine rabies. The sparseness of human (and canine) populations in very many areas, the migrations or nomadic lifestyles make the foci of infection more scattered than in North Africa, and the disease appears primarily in the urban areas or upon displacement of populations (following armed conflicts).

In the northern Sahelian zone, the prevalent situations in Ethiopia, Somalia, Sudan, Chad, Niger, Mali, Mauritania and Senegal are quite similar, even if official statistics do not permit a valid comparison. Infection of dromedaries by dogs can produce epidemiol-

ogical foci of a special nature (26). Likewise, the role of the jackal and hyena is occasionally important in some areas but as "spill-over" from canine rabies rather than being cycle-independent (27).

The Sudan-Guinea area

This includes Benin, Guinea, Nigeria, Ivory Coast, Ghana, Central African Republic, Cameroon and Kenya. The epizootic characteristics are the same in the south as in the Sahelian zone to the north, but with accidental infection of a much larger number of wild species (e.g., the large predators, monkeys, bats, rodents, insectivores, etc.). Nigeria occupies a special place in this regard, having been the source of numerous isolates of rabies-related viruses from shrews and bats (serotypes 2 and 3) and insects (kotonkan).

Equatorial Africa

This includes Congo, Gabon, Mozambique, Zaire, Uganda, and Tanzania. Here, rabies is especially conspicuous in urban and peri-urban areas. This is perhaps due to the concentration of dogs in these areas, or to the absence of epidemiological surveillance outside these same areas. Explosive epidemics can, nevertheless, erupt over the whole area (e.g., Tanzania, southern Kenya, Uganda). An epizootic of canine rabies has been observed in Gabon since 1985 (P. Sureau, personal communication): current studies indicate that conventional vaccines provide less than effective protection against it.

Southern Africa

This includes South Africa, Angola, Botswana, Namibia, Zambia, Zimbabwe. Although it is canine rabies that presents the greatest danger to man, the situation is complicated by the existence of independent cycles in some species of wild carnivores (mongooses in South Africa, jackals in Zambia and Zimbabwe) and even herbivores (kudus in Namibia) (see above). In other respects, the existence of rabies-related viruses (e.g., in the southern province of Natal and in Zambia) complicates the fight by vaccination, which is ineffective against serotypes such as Mokola (serotype 3) (28).

4. The Effect of Prophylactic Measures: Current Trends

Prophylactic measures against rabies in Africa have been sporadic and of variable effectiveness. All are characterized by the very great

difficulty of implementation, and variability in the rigor of application, depending on the time period and country (30-32). In all African countries, sanitary prophylaxis by elimination of stray dogs runs up against an uncooperative attitude of the human population. No matter what the cultural, religious or political environment of the country, all efforts to limit the number of stray dogs have either failed or have not been applied effectively - or for long enough duration - to be effective.

Medical prophylactic measures, by vaccination of non-stray dogs, have been applied only in a very limited number of countries, and often for a rather short time period. During the period of European occupation of the continent, several colonies were able to claim rabies-free

Figure 6. Systematic vaccination of dogs in some African countries (e.g., Tunisia) has resulted in a spectacular reduction in the number of rabies cases in this species and in man.

status, either by spontaneous regression of epidemics, by successful vaccination of all dogs in the country, or by a combination of the two. This was the case notably in Southern Rhodesia (Zimbabwe) from 1913 to 1950, and in several regions of east or central Africa. Following independence of the majority of African countries, the added problems (materialistic, political, financial) have often reduced the incidence of dog vaccination to negligible proportions. Since 1980, however, several countries have heeded the directives of the World Health Organization in the fight against canine rabies. Some of these countries have undertaken widespread programs of canine vaccination, in particular Tunisia (1980-) and some administrative units of Algeria, Morocco, and Tanzania.

Current trends in the African rabies epidemic are, unfortunately, static or even spreading. Rabies still remains endemic throughout the continent, and periodic epizootic outbreaks fleetingly aggravate this situation. There were outbreaks of this sort in 1974 in Morocco and Zambia (55 and 33 human cases respectively), Tanzania in 1976 (81 deaths), Mozambique in 1978 (52 deaths), Kenya in 1980 (66 deaths), etc. In view of all the difficulties encountered in the fight against rabies within the current African context, there is no cause for hope for an early reversal of this trend.

CONCLUSIONS

1. Comparative study of the epidemiological rabies situations in the different countries of Europe, Asia and Africa reveals a great disparity depending on geographical location or country. This disparity is less evident in the virus itself (with rare exceptions in Africa) than in its vectors. Depending on whether the disease is perpetuated by dog, fox, mongoose, or bat, its characteristics (and control measures) vary considerably.

2. Evolution of the epidemiological situations also varies depending on the region. It appears well established historically that canine rabies has attained an equilibrium between vector and virus, and is no longer evolving. In contrast, the "new" (sylvatic) rabies is rapidly evolving, and may expand or regress depending on variables that are

still not understood, despite the most sophisticated predictive mathematical or informational models.

3. The future of rabies in Eurasia and Africa currently appears inclined towards a perennialization or expansion rather than a regression. Indeed, despite very important progress in the immunization of dogs, this measure is less and less applied due to limited financial resources, or to political whims, in many countries in which rabies is rampant (31,32). And in many of those in which sylvatic rabies (wild carnivores or bats) is rife, control activities remain limited because of the difficulties inherent in obtaining the required materials, their cost, or their danger. Nevertheless, the success of control measures currently being implemented in several western European countries, including programs directed towards the vaccination of wildlife, provide a measure of optimism that this situation may eventually change.

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EPIZOOTIOLOGY OF RABIES: THE AMERICAS

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ABSTRACT

The epizootiology of wildlife rabies in North America, mongoose rabies in the Caribbean, and dog and vampire bat rabies in Latin America is discussed. Particular emphasis is given to the distribution and prevalence of antigenic variants in geographically separate areas and in areas where the disease is enzootic in several host species.

INTRODUCTION

Despite improvements in vaccine quality and availability and advances in diagnostics, epidemiology and surveillance, rabies continues as a threat to human and animal populations. In parts of Central and South America rabies in dogs has increased as rapidly as human populations have grown, and while effective vaccination and animal control programs have eliminated dog rabies in the United States and Canada, the disease there continues to cyclically increase and decrease in a variety of wildlife hosts.

If rabies is to be eventually eradicated or brought under control in these countries, it will be through an understanding of the epizootiology of the disease. This must include knowledge of the relationship which exists between the rabies virus and the host animal (factors such as species differences in susceptibility to infection and tissue tropism, the length of the incubation and morbidity periods, and the clinical syndrome) and of the relationship which exists between that animal and its environment (factors such as population dynamics and interactions between animal species).

J.B. Campbell, K.M. Charlton (eds.), RABIES.

Recent advances in genetic engineering have resulted in new and promising approaches to animal vaccination. As field application of these vaccines becomes likely, it is now more important than ever that we re-examine what is known about the epizootiology of rabies and outline those areas in which more information is needed.

RABIES IN CANADA AND THE UNITED STATES

Although 4 decades of successful vaccination and pet animal control programs have reduced canine rabies from more than 8,000 cases per year to a few hundred cases per year, rabies continues in the United States and Canada to be enzootic in wildlife species. From 6-8,000 sylvatic cases are reported each year, and the resulting annual rabies prophylaxis comprises 80-90,000 doses of human vaccine and 20-30 million doses of domestic animal vaccine (1,2).

This picture is unlikely to change without the development and implementation of wildlife rabies control programs on a scale comparable to that which was used against domestic animal rabies. Many of the epidemiologic methods used in assessing disease trends and the efficacy of control measures in domestic animals, however, are inadequate for the complex multi-species involvement that characterizes sylvatic rabies. In addition to an accurate assessment of the geographic distribution of cases, appropriate intervention here will require knowledge of the incidence of rabies in each of several different wildlife host species, and the potential within an outbreak area for both intra- and inter-species transmission of virus. As wildlife rabies increased in the 1960s, efforts were made by several groups to gather this information. One of the most productive has been the nationwide rabies surveillance system. Although a passive surveillance (only those animals that have been observed to have human or domestic animal contact and are submitted for laboratory diagnosis are included), the system has provided much information on the patterns of disease in each wildlife species.

One of the first observations to be made from data collected in this manner was that large numbers of cases are reported in 1 major host species in certain areas while the disease is reported only rarely in this species in other areas. For example, of 634 cases of rabies in

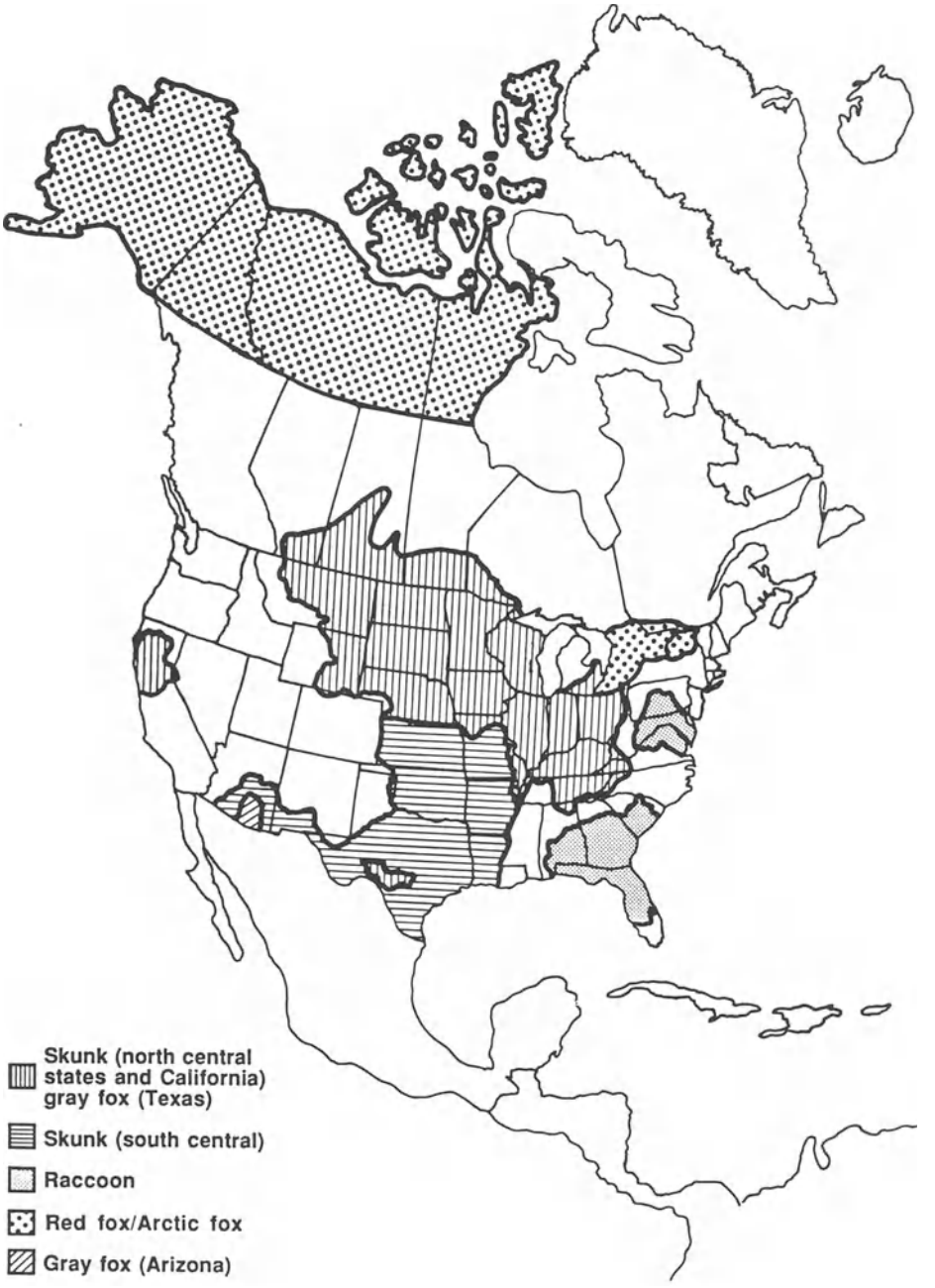

Figure 1. Distribution of antigenic variants of rabies: United States and Canada.

terrestrial wildlife in Maryland in 1986, 588 occurred in raccoons. The remaining 46 cases were distributed among 5 species. In contrast, of the 376 cases of rabies in terrestrial wildlife in the state of California in 1986, 368 rabid skunks were reported and no rabid raccoons (1).

Compartmentalization of the disease in this manner has resulted in the predominance of rabies in a single host species in several areas of Canada and the United States (Fig. 1, Table 1). In 1986 75% of 3,565 reported skunk rabies cases occurred in a large area that extends from southern Alberta, Saskatchewan and Manitoba, Canada, across the central United States to the Rio Grande River and a separate skunk rabies area of California. Ninety-nine percent of 1,609 rabid raccoons reported in 1986 occurred in the southeastern and mid-Atlantic United States. Of

Table 1. Reported Animal Rabies Cases in the United States and Canada by Major Host Species and Enzootic Area, 1986

Enzootic: Host animal/ Area	Fox	Skunk	Raccoon	Bat	Other wildlife	Domestic animals	Total
Arctic fox *Alopex lagopus*/ Northwest Territories, Alaska	35	0	0	0	1	0	36
Red fox *Vulpes fulva*/ Ontario, Quebec, New York	1,720	741	33	92	56	780	3,424
Skunk *Mephitis mephitis*/ Saskatchewan, Manitoba, California, Central U.S.A.	63	2,564	9	505	13	397	3,551
Raccoon *Procyon lotor*/ Mid-Atlantic, Southeast U.S.A.	96	191	1,567	161	26	110	2,151

Source: Centers for Disease Control, Rabies Surveillance Annual Summary, 1986.

the 1,915 fox rabies cases reported in 1986, 90% occurred in southern Ontario and Quebec and northern New York and the small number of rabies cases reported each year from Alaska and the Northwest Territories (20 in Alaska and 16 in the Northwest Territories in 1986) occur almost exclusively in arctic foxes (1).

Within these areas, the phenomenon of single-species involvement is almost universal, and the cases that occur in other animals are generally regarded as "spill-over" or accidental infection from the major reservoir animal. The reason for the association of a particular wild species with the disease in a given geographic area remains a mystery. Although ecologic isolation between species may be a factor preventing inter-species transmission, compartmentalization of the disease within 1 animal species does not seem to be caused by lack of sufficient numbers of other species. Early studies of skunk and fox population densities in relation to rabies had shown that there were approximately as many skunks in the fox rabies areas of the eastern United States as there were in the skunk rabies areas of the Midwest and, conversely, that there were as many foxes in parts of Wisconsin and Minnesota as in New York (3,4).

Several experimental studies have suggested that differences in species susceptibility may be important in compartmentalization (5,6). For example, comparisons of the pathogenesis of the disease in skunks and foxes inoculated with viruses isolated from the respective species have shown quantitative differences in susceptibility and response to infection (Table 2). While foxes and skunks appeared equally susceptible to infection with virus isolated from the salivary gland of a naturally infected skunk, foxes were 100 times more susceptible than skunks to infection with virus isolated from a fox salivary gland and foxes given large lethal doses of either virus were less likely to have infectious salivary virus than those given smaller virus inocula. Moreover, skunks inoculated with either virus were more likely than foxes to have infectious saliva, generally excreted more virus in their saliva, and virus excretion was not affected by the amount of virus used to infect the animals. The assumption made from these studies is that although the titers of virus excreted by rabid foxes were in the optimum range for infection of other foxes (8 of 10 foxes excreting

Table 2. Comparative Results of Inoculation of Rabies Virus in Foxes and Skunks

Approx. virus dose ($MICLD_{50}$)[a]	Rabies deaths/ number inoculated				Saliva virus isolated/ number dying of rabies			
	Skunk virus		Fox virus		Skunk virus		Fox virus	
	Skunks	Foxes	Skunks	Foxes	Skunks	Foxes	Skunks	Foxes
100,000	6/6	5/6	7/7	n.d.	5/6	0/5	5/7	n.d.
10,000	6/6	5/5	6/6	6/7	3/6	3/5	6/6	1/6
1,000	6/6	6/6	5/6	7/7	4/6	1/6	4/5	1/7
100	5/6	5/6	0/6	4/7	3/5	4/5	0/0	2/4
10	2/5	2/5	n.d.[b]	7/7	0/2	1/2	n.d.	6/7

[a] mouse intracerebral 50% lethal dose.

[b] n.d. = not done.

Source: Sikes (5) and Parker and Wilsnack (6).

virus after inoculation with fox virus excreted less than 100 $MICLD_{50}$), only a small percentage of infected foxes would emit enough virus to infect skunks. If the incidence of fox rabies in an area remains high for long periods and a sufficient number of skunks are exposed however, it is likely that an infected skunk would emit sufficient salivary virus to effect a secondary intra-species cycle of transmission. Conversely, although one might expect to see an occasional fox infected in an area characterized by a predominance of skunk rabies, the massive amounts of virus in skunk saliva (the mean titers of saliva excreted by skunks infected with skunk virus ranged from 20-200,000 mouse LD_{50}) would be likely to kill an infected fox before virus was excreted in its saliva.

These studies also suggest that certain virus isolates have differences in pathogenicity for different host species, related at least in part to the animal hosts in which the virus has been passed. The difference noted in Table 2 in the comparative susceptibility of foxes and skunks to virus isolated from foxes is one example.

Table 3. Reaction Patterns[a] of Virus Isolates from Major Terrestrial Rabies Enzootic Areas

Major host/ area/ year	Hybridoma number[b]																	# of isolates
	1	2	3	7	10	11	12	15	16	17	18	19	T	P	5	8	13	
Red fox/ Ontario, Quebec New York 1978-1987	ø	+	+	+	+	+	+	ø	ø	+	ø	ø	+	+	+	+	+	37
Arctic fox/ Alaska, NWT 1984-1987																		9
Raccoon/ Mid-Atlantic USA 1978-1987	ø	+	+	+	+	+	+	+	+	+	+	+	+	ø	+	+	+	76
Raccoon/ Southeastern USA 1978-1987																		95
Skunk/ South-central USA 1975-1987	ø	ø	+	ø	+	ø	ø	+	+	+	+	+	ø	ø	+	+	+	105
Gray fox/ Arizona 1970-1987	+	+	ø	+	ø	+	+	+	+	o	ø	+	+	ø	+	+	+	15
Skunk/ Manitoba Saskatchewan North-central USA 1978-1987	+	+	+	+	+	+	+	+	+	+	ø	+	+	ø	+	+	+	60
Skunk/ California 1974-1983																		6
Gray fox/ Central Texas/ 1983-1986																		13

[a] Symbols: ø = no reaction; o = diminished reaction with 10x less dilute antibody; + = positive reaction

[b] In previous publications (refs. 15,16,19) these Mab-Ns have been indicated in the following manner: 1 = 3-1; 2 = 8-2; 3 = 11-1; 5 = 22-3; 7 = 24-1; 8 = 24-10; 10 = 52-2; 11 = 61-1; 12 = 62-4; 13 = 71-2; 15 = 97-3; 16 = 97-11; 17 = 141-1; 18 = 143-1; 19 = 146-3; T = Tu 187-5; P = Tu P41.

Until recently, more detailed study of rabies variants has been limited by the lack of laboratory methods for identifying them. The use of monoclonal antibodies to detect antigenic variation now permits the characterization of virus isolates from separate species-defined enzootics and has increased interest in the role virus strain differences may play in compartmentalizing sylvatic rabies. Numerous investigators have used monoclonal antibodies to identify and group rabies variants (7-22). Antigenic differences in both nucleocapsid proteins and glycoproteins have been useful in this regard but for practical application in epidemiology, monoclonal antibodies to the N protein (Mab-N) are the easiest to use. The N protein (see Tordo and Poch, this volume) is one of the most abundant proteins produced in a rabies infection and is easily detected in the brains of naturally infected animals, thus minimizing the laboratory manipulation required for analysis. By recording the immunofluorescence reaction of a panel of Mab-Ns, characteristic reaction patterns with virus from a given outbreak area can be defined, and antigenic differences in isolates from geographically separate outbreaks can be identified. Spill-over infection from the major reservoir animal can then be estimated by testing isolates from a variety of animal species that are only sporadically infected in these species-defined enzootic areas.

The results of such tests on 427 isolates (15,19,21) are shown in Table 3. These isolates could be identified as belonging to 1 of 4 groups, and the geographic distribution of these groups generally corresponded to the separate species-defined enzootic areas recognized by surveillance data (Table 1, Fig. 1). A single reaction pattern was found for all virus isolates collected during 10 years from terrestrial animals in areas of the northeastern United States and southern Ontario and Quebec where red foxes are the predominant rabid host species. An identical reaction pattern was observed in isolates from arctic fox rabies areas of Alaska and the Northwest Territories. This pattern was not found in any other area of North America.

Another distinctive pattern characterized 76 isolates from rabid terrestrial animals in the raccoon rabies enzootic area of the mid-Atlantic states. This same pattern was seen in 95 isolates from

raccoon rabies areas of the southeastern states but was not found elsewhere in North America.

Rabies virus isolates from terrestrial species in the large skunk rabies band extending through the central United States and adjoining areas of Alberta, Saskatchewan, and Manitoba could be divided into 2 reaction groups. One group comprised 60 isolates from the skunk rabies area of the north-central states and contiguous areas of Canada. An identical pattern was found in skunk rabies isolates from California. The second reaction group comprised 105 isolates from skunk rabies areas of the south-central United States.

The antigenic pattern characterizing isolates from a given enzootic area remained constant when isolates were collected over a period of 8 to 18 years and when isolates were collected from either the major host species or other species suspected to have become infected subsequent to exposure to the major host species. For example, the intensity of the raccoon rabies enzootic in Maryland, coupled with the human population density in this area, resulted in detecting rabies in a variety of animal species within the raccoon rabies enzootic area of the state. (In 1986, in addition to 588 raccoons, rabies was diagnosed in 18 cats, 3 cows, 1 horse, 1 sheep, 12 skunks, 25 foxes, 7 woodchucks, 1 squirrel, and 1 deer.) Mab-N tests were performed on virus isolates from 11 cats, 1 horse, 1 squirrel, 1 cow, 2 woodchucks, 3 skunks, and 1 fox found rabid in the raccoon rabies enzootic area of the state. The antigenic patterns of virus from all of the isolates tested, regardless of species of origin, were identical and characteristic of the variant found in infected raccoons in this area (15). The characteristic reaction pattern of isolates from this area also remained stable when isolates were passaged *in vitro* in a variety of animal cell culture lines or laboratory animals (personal communication, C.E. Rupprecht).

Although identical reactivity with a panel of Mab-Ns cannot be construed as indicating identical virus strains and isolates belonging to some of the larger groups may be subgrouped as more Mab-Ns are added to the panel (20), some interesting observations can be made when the geographic distribution of virus strains with identical reaction

patterns is compared with what is known from surveillance data describing the movement of rabies into these areas.

For example, the observation of the same rabies variant in virus isolates from New York and Maine and Ontario and Quebec is in agreement with surveillance data from the early 1960s which reported the movement of rabid foxes from Ontario across the St. Lawrence River into the northeastern United States (23,25) and the suggestion that enzootic rabies in the northeastern United States is the continuation of an outbreak that began in red foxes in Canada in the 1950s (24). That this same variant is found in areas of arctic fox rabies in Alaska and the Northwest Territories supports Tabel's account of the movement of rabies from arctic foxes in polar regions of North America to become established in red foxes in the more temperate regions of Ontario and Quebec (25).

The finding of the same antigenic pattern associated with isolates from the established raccoon rabies areas of the southeastern states and the more recently developed raccoon outbreak area of the mid-Atlantic is also compatible with surveillance and epidemiologic data. In 1980 a new outbreak of raccoon rabies was recognized in the mid-Atlantic states along the Virginia - West Virginia border. From a single reported case in 1977, the outbreak expanded to include Maryland, Pennsylvania, Delaware and the District of Columbia and to comprise a thousand or more cases each year (1,26). It is suspected that rabies was introduced into this area as a result of transporting infected raccoons from enzootic areas of the southeastern United States. Rabies was diagnosed in 2 raccoons that were part of a shipment from the Southeast to private hunting clubs in the mid-Atlantic region (27), and thousands of raccoons are imported into this area for hunting purposes each year.

Another interesting surveillance observation, also supported by the Mab-N data, is the recognition in the early 1960s of increased activity in 2 well separated skunk rabies areas: an older well established focus in Minnesota - Iowa and a newly emerging epizootic in Texas (28). The expansion of these 2 areas during the next 10 years resulted in their merger in parts of Missouri and Arkansas (1,29). Mab-N analysis of isolates from what now appears as a single large

skunk rabies enzootic area in the central United States and contiguous regions of Canada reveals 2 different variants (Fig. 1), with both variants present in Missouri and Arkansas.

In addition to lending support to established epidemiologic observations, the identification of antigenic variants associated with separate species-defined enzootic areas can also contribute to new epidemiologic studies. For example, a clustering of 10-20 rabies cases in gray foxes in eastern Arizona and a similar clustering of fox rabies cases in central Texas (Fig. 1, Table 1) are observed each year (1). It had not been possible to determine by surveillance of disease incidence whether rabies in these foxes was the result of interspecies transfer of virus from the active skunk rabies enzootic in these states or whether these cases were the result of an independent cycle of rabies transmission within the fox population.

Monoclonal antibody analysis of isolates from these areas suggests independent cycles in these 2 species (Table 3). The reaction pattern of 15 rabies isolates collected over a period of 18 years from an area of Arizona that consistently reports a few cases of rabies in gray foxes each year differed markedly from that of other isolates from the surrounding skunk rabies area. A similar observation was made after analyzing isolates from gray foxes in 3 counties in central Texas. These viruses could be easily distinguished from isolates from 26 other Texas counties where skunk rabies predominates. The antigenic differences in virus isolates from the 2 species did not arise from a single instance of spill-over passage of "skunk" virus in gray foxes: when an occasional rabid gray fox is found in skunk rabies areas outside these small foci of fox rabies, the reaction pattern of the virus isolated from the fox is identical to that of virus normally associated with animals from the skunk rabies enzootic area (19).

The finding of antigenically different variants of rabies virus in overlapping outbreaks affecting different animal species has interesting implications for both wildlife disease control efforts and epidemiologic studies. At its simplest, this finding would suggest that any wildlife disease control measures proposed for these areas must be directed to both species. Eliminating skunk rabies in these areas, although reducing the number of cases from several hundred to several

dozens per year, would probably not eradicate terrestrial rabies from there. Additionally, an area with easily identifiable separate cycles of virus transmission would also provide a unique setting for epidemiologic studies. Studies of animal population dynamics and fox-skunk interactions in these areas could reveal much about the role of animal/environmental relationships in interspecies disease transmission. Much could also be learned of virus-host relationships by studies of species differences in susceptibility and response to infection with these 2 rabies variants, studies similar to those conducted by Sikes and Parker that are described in Table 2.

Although in these 2 instances independent transmission cycles affecting 2 species in a single geographic area were conveniently accompanied by antigenic differences, other enzootic areas exist where surveillance suggests more than 1 cycle of rabies transmission which Mab-N data cannot verify. The increased involvement of striped skunks in the red fox rabies areas of Canada and the northeastern United States illustrates this. Fox rabies entered southern Ontario in 1956 (25), the first few cases of skunk rabies occurred in 1957, and by 1960 the striped skunk was the second most important wildlife vector in this area, responsible for 30-50% of reported rabies cases (25). By Mab-N testing, the same strain of virus was isolated from both species (20). This epizootic spread to red foxes in adjacent areas of New York (1), with the proportion of fox to skunk cases similar to that observed in southern Ontario. Similarly, in addition to 409 rabid raccoons, 101 rabid skunks were reported in the raccoon rabies enzootic area of Pennsylvania (1). Although the high incidence of disease in skunks in these areas would suggest intraspecies transmission of rabies, all virus isolates from these areas reacted identically with Mab-Ns. Additionally, these viruses were identical in their reaction with a panel of monoclonal antibodies that recognize 40 different antigenic sites on the rabies glycoprotein (30).

These laboratory findings and the absence of rabies in skunks before the appearance of rabid foxes (or raccoons) suggests intraspecies transmission of rabies was accompanied by subsequent adaptation in a different host and indicates an area of needed research. Discovery and molecular characterization of epitopes unique to a species-

associated rabies variant may reveal important information about what genetic changes, if any, may specify adaptation to different host species. The epitope reactive with the Mab-N P41 may be one such region of the N gene. Mab-N P41 reacts specifically and exclusively with viruses from the arctic and red fox rabies areas of the northern hemisphere (18) (Table 3). Dietzschold *et al.* (31) have characterized peptides from conserved and variable regions of the N gene which may be associated with important biological functions of the virus. Other regions on the N protein and on other viral proteins may also be important in adaptation to a particular ecologic niche.

Despite the obvious limitations of epidemiologic mapping of variants on the basis of antigenic difference, the observation of characteristic antigenic patterns for rabies isolates from the terrestrial species-defined enzootic areas can be used to predict certain epidemiologic relationships. One of the most important applications of this technique is assessing the contribution of rabid insectivorous bats to the disease in terrestrial animals.

Since the recognition of bats as rabies hosts in 1953 (32), rabid bats have been reported in all of the continental United States and in the lower Canadian provinces (1). Although human infection as a result of contact with infected bats is well-documented (33) and it is possible to experimentally infect several species of animals with virus from naturally infected bats (34-36), the contribution of bats to enzootics in terrestrial animals is unknown. Infected bats are suspected as a source of rabies infection in terrestrial mammals, particularly as the source of rabies that has suddenly appeared in areas or species that previously were unaffected (37). Geographic associations have been made for the distribution of caves and the occurrence of rabies in foxes (38,39), and rabies-infected carnivores have been trapped adjacent to caves occupied by great numbers of Mexican free-tailed bats (40).

On the other hand, there is also evidence from surveillance data that infected bats do not play an important role in terrestrial rabies enzootics. Several states consistently report rabies in bats while remaining free of recurring cases in domestic animals and terrestrial wildlife (1).

Table 4. Results of Tests for Rabies Virus in Suspect Bats from 4 States[a]

Species of Bat	Bat species positive for rabies by state of origin			
	New York	Colorado	Arkansas	California
Eptesicus fuscus	282	82	3	19
Tadarida brasiliensis mexicana	0	1	0	88
Lasiurus cinereus	20	50	0	82
Lasiurus borealis	16	3	9	2
Other species[b]	45	23	0	55
Unidentified	18	7	7	42
Total positive	381	166	19	288

[a] Bats were submitted for rabies testing in the following years: New York, 1975-84; Colorado, 1981-84; Arkansas, 1982; California, 1954-65.

[b] Other species include 1 *Macrotis waterhousii*, 23 Myotis species, 13 *Lasionycteris noctivagans*, 6 *Pipistrellus hesperus*, 2 *Plecotus townsendii*, and 10 *Antrozous pallidus* from California; 9 *Lasionycteris noctivagans*, and 14 Myotis species from Colorado; 44 Myotis species and 1 *Lasionycteris noctivagans* from New York.

Source: Constantine (43) (California); McChesney (45) (Arkansas); C. Trimarchi (New York) and J. Emerson (Colorado), personal communications.

Although extremely important as a factor in the success of wildlife rabies control efforts, epidemiologic proof of the consequence or inconsequence of natural transmission of rabies from infected bats to terrestrial species is lacking. Several recent epidemiologic studies have concentrated on the use of monoclonal antibodies to address the relationship between rabies in bats and in terrestrial animals (19,22).

The approach used to characterize isolates from bat species in epidemiologic studies necessarily differs from that used for the study of terrestrial rabies enzootics. Although geographically separate outbreak areas allow the comparison of variants from terrestrial animals on the basis of their collection site, no geographic outbreak areas have been defined for bat rabies. In fact, infected bats of

Table 5. Reaction Pattern of Virus Isolates from Bat Species

Isolate	Hybridoma number																	# of isolates
	1	2	3	7	10	11	12	15	16	17	18	19	T	P	5	8	13	
Tadarida brasiliensis mexicana	+	+	+	+	+	+	+	ø	ø	+	ø	ø	+	ø	+	+	+	20/20
Lasiurus cinereus	o	+	+	+	+	+	+	ø	o	+	ø	ø	ø	ø	+	+	+	16/18
Lasiurus borealis	ø o	+	+	+	+	+ o	ø	ø	o	+	ø	ø	ø	ø	+	+	+	17/18
Eptesicus fuscus fuscus	ø	+	+	+	+	+	+	ø	+	+	ø	ø	+	ø	+	+	+	27/35
Eptesicus fuscus fuscus	ø ø	+ +	ø ø	+ +	ø ø	+ +	+ +	ø ø	+ +	ø ø	ø ø	ø ø	+ +	ø ø	o o	ø ø	+ ø	6/35 2/35
Eptesicus fuscus pallidus	+ ø ø ø ø ø ø	+ + + + + + +	ø ø ø ø ø ø ø	+ + + + + + +	ø ø ø ø ø ø ø	+ + + + + + +	+ + + + + + +	ø ø ø ø ø + ø	+ + ø ø + + +	ø ø ø ø ø ø ø	ø + + + + + ø	ø + + + + + ø	+ + + + + + +	ø ø ø ø ø ø ø	o o o o o o o	ø ø ø ø ø ø ø	+ ø ø + o + +	2/15 2/15 3/15 4/15 1/15 2/15 1/15

For explanation of symbols, see legend to Table 3.

migratory species such as *Lasiurus cinereus* have been found throughout the natural range of these species that extends for thousands of miles (41,42). Two lasiurine bats which were collected in Iowa and eventually died with naturally acquired rabies infections, developed the disease when they normally would have been south of Texas (43). The number of affected species (30 of the 39 indigenous species (44)) also makes the selection of isolates for laboratory study difficult. The bat species are widely variant anatomically and physiologically, as well as in their habits and in their geographic distribution. Even the 2 sexes may have extremely different habits that could affect their susceptibility to rabies infection. Concomitantly, incidence of the disease varies widely in different species and in different geographic areas.

Were each of the bat species to be implicated in the disease in terrestrials, each would present a separate problem in epidemiology as it would in disease control. However, surveillance data have suggested that rabies may be enzootic in only a few of the indigenous species with other species affected only by spill-over from the major host species (Table 4) (20,41), and most cases throughout Canada and the United States are reported in 4 species (*Lasiurus borealis, Lasiurus cinereus, Eptesicus fuscus*, and *Tadarida brasiliensis mexicana*) (41,43,45).

Mab-N typing has to some extent supported these surveillance observations. When 114 rabies isolates from these species were examined, differences in reaction with a panel of Mab-Ns permitted the formation of 6 different reaction groups (Table 5). The reaction patterns of 20 virus isolates from Mexican freetail bats (*Tadarida brasiliensis mexicana*) collected in New Mexico, Colorado, Arizona, Nevada and Texas were identical to each other, and this pattern of reactivity could be used to distinguish them from the other bat species. A second reactivity pattern characterized isolates from rabid lasiurine species. Slight but consistent differences were found between isolates from red bats (*Lasiurus borealis*) and hoary bats (*Lasiurus cinereus*). Two different reactivity patterns were observed in (*Eptesicus fuscus*) rabies isolates from the eastern United States and Canada. Of the 35 Eptesicus bats examined, 27 were observed to have an identical pattern. A different pattern was found in 8 other Eptesicus bats, some collected within days and from within the same county as bats in the first group. A pattern similar to this minor Eptesicus pattern was found in *Eptesicus fuscus pallidus* bats in the Western states, but reactivity with certain Mab-Ns varied such that 7 different patterns were found in the 15 isolates tested. Although no single pattern was found to be characteristic of all isolates from this species, they could be easily distinguished from the variants associated with the disease in the 3 other bat species examined.

Examination of 11 isolates from Myotis species revealed a variety of reaction patterns (Table 6). No reaction pattern common to all isolates was found and furthermore, the pattern identified in most of these isolates was characteristic of the variant associated with the

Table 6. Reaction Pattern of Virus Isolates from Bat Species: Myotis Species

Isolate	Hybridoma number																	# of isolates
	1	2	3	7	10	11	12	15	16	17	18	19	T	P	5	8	13	
Myotis lucifugus New York	ø o	+	+	+	+	+	+	ø	ø	+	ø	ø	+	ø	+	+	ø	2
Myotis lucifugus New York	ø	+	+	+	+	+	+	ø	ø	+	ø	ø	ø	ø	+	+	ø	2
Myotis lucifugus New York	ø	+	+	+	+	+	ø o	ø	o	+	ø	ø	ø	ø	+	+	+	2
Myotis keenii New York, Maryland	ø	+	+	+	+	+	ø	ø	o	+	ø	ø	ø	ø	+	+	+	2
Myotis evotis Colorado	o	+	+	+	+	+	+	ø	o	+	ø	ø	ø	ø	+	+	+	1
Myotis ? Arizona, Nevada	ø	+	ø	+	ø	+	+	+	+	ø	+	+	+	ø	o	ø	ø	2

For explanation of symbols, see legend to Table 3.

major rabid bat species in the area. For example, 3 different reaction patterns were found in virus isolates from 6 little brown bats (*Myotis lucifugus*) in New York and the reaction pattern of 2 of these was identical to that of *Lasiurus borealis* bats. The reaction patterns of 2 *Myotis keenii* bat isolates was also like that found in red bats in New York. A single specimen from a *Myotis evotis* bat in Colorado was identical to 14 isolates from hoary bats in Colorado. The virus found in 2 Myotis isolates in Arizona and Nevada were indistinguishable from *Eptesicus fuscus* isolates in this area.

This diversity of antigenic types suggests that no separate cycle of rabies exists in Myotis species but that their infection is the result of spill-over from other bat species. These bats, although some of the most abundant species in North America, are only occasionally found rabid. For example, although *Eptesicus fuscus* and *Myotis luci-*

Table 7. Virus Isolates from Terrestrial Species Infected with Virus Typical of Infected Bat Species

Animal	State	Year	Characteristic Mab-N reaction pattern
Cow	Pennsylvania	1982	*Eptesicus fuscus fuscus*
Fox	Oregon	1982	-*
Fox	New York	1983	*Eptesicus fuscus fuscus*
Horse	New Hampshire	1983	*Eptesicus fuscus fuscus*
Cow	Virginia	1983	-
Cat	California	1983	*Eptesicus fuscus pallidum*
Fox	New York	1984	*Eptesicus fuscus fuscus*
Fox	New York	1984	*Eptesicus fuscus fuscus*
Fox	New York	1984	*Lasiurus borealis*
Cat	Oregon	1984	*Eptesicus fuscus pallidum*
Cat	Colorado	1985	-
Cat	New Mexico	1985	*Tadarida brasiliensis mexicana*
Fox	Michigan	1986	*Eptesicus fuscus pallidum*
Fox	Rhode Island	1986	*Eptesicus fuscus fuscus*
Cat	Utah	1986	-
Cat	New York	1986	*Eptesicus fuscus fuscus*
Sheep	Illinois	1986	*Lasiurus borealis*
Horse	New York	1986	*Eptesicus fuscus fuscus*
Horse	Nevada	1986	*Eptesicus fuscus pallidum*
Fox	New York	1987	*Lasiurus borealis*
Fox	New York	1987	*Lasiurus borealis*
Fox	New York	1987	*Lasiurus borealis*
Skunk	Colorado	1987	*Eptesicus fuscus pallidum*

* Reaction pattern not characteristic of virus associated with the 4 most commonly infected bat species, but has been found in a small number of isolates from other bat species.

fugus are both prevalent bat species in Ontario, 43 of 534 *Eptesicus fuscus* bats submitted for rabies testing in 1984 were positive, while only 1 of the 179 bats submitted from Myotis species was positive (20).

None of the reaction patterns that characterize bat rabies was represented in the patterns for the major terrestrial rabies enzootics. This supports the premise of many epidemiologists that rabies in bats exists as an enzootic largely independent of the cycle in terrestrial animals and that bats are not an important maintenance source of infection for the currently recognized enzootics in terrestrial wildlife populations.

Evidence has shown, however, that rabies infection in terrestrial animals does occur as a result of contact with infected bats. Several areas in North America, which although free of enzootic rabies in terrestrial species and reporting only a few bat rabies cases each year, report an occasional case of rabies in a terrestrial animal (46,47). These animals may have become infected by contact with infected bats, by immunization with a live attenuated rabies vaccine, or by contact with a rabid animal in an enzootic area and subsequent transport to a rabies-free area. Comparison of the reaction patterns of viruses from 23 of these isolated cases of rabies (Table 7) with those of the 5 major terrestrial types (Table 3), the known bat types (Tables 5 and 6) and the patterns of the 3 attenuated rabies vaccines (Table 8) suggested that these animals had been infected by contact with rabid insectivorous bats. In 2 cases (a rabid cow in Pennsylvania in 1982 and a rabid horse in New York in 1986), epidemiological investigations had revealed possible contact with infected bats. A farmer had reported removing a bat from the cow 2 months earlier (46) and a rabid *Eptesicus fuscus* bat had been found in a shed near the horse's stable (personal communication, C. Trimarchi). Although the bat in Pennsylvania was not submitted for rabies testing, the bat in New York was tested, and Mab-N analysis revealed a pattern identical to that found in the horse.

The occurrence of most of these cases in cats and foxes would suggest that additional cases of bat-to-terrestrial animal rabies transmission might also be found among the several hundred cats and foxes reported rabid each year. Since almost all of these animals are

Table 8. Reaction pattern of vaccine strains

Vaccine	Hybridoma number																
	1	2	3	7	10	11	12	15	16	17	18	19	T	P	5	8	13
ERA	+	+	+	+	+	+	+	+	+	+	+	+	ø	ø	+	+	+
HEP	+	+	ø	+	ø	+	+	+	+	ø	ø	+	+	ø	+	ø	+
LEP	+	+	+	+	+	+	+	+	+	+	ø	+	+	ø	+	+	+

For explanation of symbols, see legend to Table 3.

reported in areas enzootic for rabies, however, investigators have assumed that their infection was the result of contact with the predominant terrestrial host species in that area. Mab-N analysis of numerous fox and cat cases should indicate whether this is so and could give a rough estimate of the contribution of infected bats to the maintenance of enzootic rabies areas.

When 136 cat and fox isolates from established enzootic areas were examined, 2 of 66 cat isolates and 1 of 70 fox isolates examined suggested contact with infected bats (48). A viral isolate from a cat found rabid in an area of Georgia enzootic for raccoon rabies was identical to viral isolates from red bats in this area and a viral isolate from a cat found rabid in an area of Texas enzootic for skunk rabies was identical to viral isolates from Mexican freetail bats in this area. A viral isolate from a rabid fox in Arizona was identical to viral isolates from *Eptesicus fuscus* bats in Arizona. The observation of only 3 of 136 cases in which transmission of rabies from bats to cats and foxes may have occurred suggests that such transmission is of low frequency and that most of the rabies in cats and foxes in the United States is the result of spill-over from the major reservoir in terrestrial species.

The ability to identify 26 cases of rabies in terrestrial animals that are evidently the result of contact with infected bats, however, suggests that the combination of careful surveillance and Mab-N based identification of virus isolates may eventually determine whether bats play a significant role in the emergence of new epizootics in terrestrial species. A recent case identified in New York illustrates this potential. In December 1983, a single case of rabies was identified in a gray fox in Dutchess County, an area that is far removed from the red fox rabies area in the northern part of the state, and had been free of terrestrial rabies for 25 years. In October 1984, 3 additional rabies cases were reported in foxes from this same area. No cases were reported in 1985 and 1986; however, 3 additional cases in gray foxes were identified in this area in late 1987 (personal communication, C. Trimarchi). Mab-N typing of these 7 isolates revealed a pattern identical to that of Eptesicus bats in 3 isolates and of *Lasiurus borealis* bats in 4 isolates. Although the small number of cases, their

time of occurrence, and the finding of 2 different rabies variants in them would suggest that enzootic conditions were not established, the possibility that such conditions may arise makes this an important area for study.

RABIES IN LATIN AMERICA AND THE CARIBBEAN

The travellers who cross the international bridge from the United States to Mexico enter a country with a different language, heritage and customs. But besides these differences they enter a country where canine and vampire bat rabies are common, a situation that extends from northern Mexico to Argentina. The continued severity of the disease in dogs 'south of the border' is reflected in the many human rabies deaths and the high rate of human vaccination; moreover, vampire bat rabies leads to hundreds of thousands of cattle deaths every year, and sporadic human cases. How different those 2 epidemiological forms are from the disease in the United States and Canada, where rabies has been virtually eliminated from dogs, but remains enzootic in skunks, raccoons, foxes, and insectivorous bats.

Canine Rabies

Even after the effect of limited reporting in some enzootic areas of Latin America is taken into account, dog rabies accounts for well over 50% of all animal rabies reported in the Americas. Hemispheric rabies surveillance begun by the Pan American Zoonoses Center (CEPANZO; Pan American Health Organization) in 1969 clearly indicates that, year after year, dogs are the animals most often reported rabid (49) and in

Table 9. Reported Rabies Cases/Year in Latin America and the Caribbean

Rabies cases	1970-79	1980-84	1985
Wildlife	215	530	348
Dogs	17,373	16,135	14,890
Cattle	3,193	4,313	3,500
Cats	887	906	436
Humans	286	308	243

Source: Pan American Health Organization, RIMSA5/INF/2, April 20, 1987.

Table 10. Human Rabies Cases, by Animal Contact, the Americas, 1970-79

Infected Animal	Number of Human Cases (%)	
Dog	1,463	(92.8)
Bat	51	(3.2)
Fox	6	(3.8)
Cat	47	(3.0)
Cattle	3	(0.2)
Rat	3	(0.2)
Skunk	2	(0.1)
Mountain lion	1	(<0.1)

Source: CEPANZO (PAHO/WHO).

Latin America and the Caribbean may account for 80% of all reported animal rabies. A summary of the Center's report for 1970-1985 is shown in Table 9. Mexico reports the largest number of cases, with 10,036 cases of canine rabies reported in 1985 (50; see also Larghi *et al.*, this volume). It must be added that the efficiency of reporting depends on a given country's surveillance system; those countries with extensive diagnostic laboratory networks usually report more rabies than those countries of similar population with a less developed system.

The predominance of canine rabies has not changed much in most Latin American countries. In 1986, for instance, Mexico reported that 93.5% of its 9,088 animal rabies cases were in dogs (1). Dogs are also responsible for almost all human rabies deaths. In the 1970-1979 decade 92.8% of reported human rabies in the hemisphere were the result of contact with rabid dogs (Table 10) (51). The same pattern of canine predominance has been noted in tabulating the animals responsible for human post-exposure rabies vaccination.

Almost no data are available on what percentage of human rabies deaths in areas of enzootic canine rabies might go undiagnosed. There is, however, one remarkable study of 1,547 post-mortem specimens from persons who died of unknown causes in and around Cali, Colombia, between 1962 and 1966 (52). The examination of brain material from those specimens showed that an astounding 1.7% of all the deaths had

Table 11. Rabies Deaths Diagnosed in Autopsy Material from the Universidad del Valle Hospital, Cali, Colombia (Persons Over 1 Year of Age), 1962-66

Year	No. of autopsies	Rabies cases	% of rabies cases
1962	375	2	0.5
1963	364	6	1.6
1964	348	8	2.3
1965	233	5	2.1
1966	276	6	2.2
Total	1,596	27	1.7

Source: Sanmartin *et al.* (52).

actually been due to rabies, with the rate varying from 0.5% to 2.3% in the various years studied (Table 11). The authors concluded that: "This alarming percentage of rabies cases in autopsies done in our school of medicine is a clear reflection of the situation that exists in our community and which we are sure is no different from that in the rest of the country. We believe that a careful examination of pathological material from other teaching centers...would show that the situation in Cali really is not unique."

Canine rabies is enzootic in most of Latin America although its epizootiology has not been closely studied. The few studies on canine rabies have dwelt on the control of outbreaks by vaccination and elimination of stray dogs (53,54). There is apparently no information on the percentage of dogs with 'naturally acquired' antibodies in enzootic areas in Latin America similar to the 19% of unvaccinated dogs with antibody found in Chiang Mai province, Thailand (55), nor are there any data on incubation periods in naturally infected dogs. It is known, however, that experimentally infected dogs with longer incubation periods are more likely to have virus in their salivary glands and that the titers in those glands are higher than those of dogs dying of rabies after a short incubation period (56). As a corollary to those experimental results, we examined the salivary glands of dogs naturally infected with rabies in Mexico City in 1978, and almost all were infective, with high titers (Table 12), suggesting that the incubation

Table 12. Titers of Rabies Virus in the Submaxillary Salivary Glands of Naturally Infected Dogs in Mexico, D.F., 1978[a]

Dog #	Titers ($log_{10}MICLD_{50}$/ ml)[b]	
	LSG[c]	RSG[c]
5952	5.5	5.7
5919	5.5	5.3
6089	4.7	5.0
6285	4.5	3.7
6119	5.5	6.3
5951	5.7	6.3
6144	4.5	4.3
6189	<3.0	<3.0
5953	6.3	6.0
6286	5.7	5.7
6145	5.3	4.3
5694	5.7	5.7

[a] 11/12 dogs had positive salivary glands.

[b] Mouse intracerebral 50% lethal dose.

[c] LSG = left salivary gland; RSG = right salivary gland.

Source: Martell, Shaddock and Baer, unpublished data.

periods in those dogs had been long. Since long incubation periods in experimentally infected animals are usually the result of inoculation with low doses of challenge virus (56), this further suggests that those street dogs were exposed to low doses of virus.

When canine rabies control programs are initiated, the number of canine cases falls rapidly (see Larghi *et al.*, this volume). A recent example is that of Argentina (57) where 6,567 rabid animals were reported in 1976, almost all dogs. The number of rabies cases dropped steadily over the next 10 years in the face of continued canine vaccination of 2,000,000 doses annually (Table 13) and in 1986 only 1 focus of canine rabies (40 cases) remained. During that same period human rabies deaths also decreased markedly (Table 14) (57).

Table 13. Animal Cases in Argentina, 1976-86

Year	Dogs	Cats	Cattle	Wildlife	Total
1976	4,811	388	1,360	8	6,567
1977	2,334	253	131	6	2,724
1978	1,115	132	296	6	1,549
1979	1,033	95	144	1	1,273
1980	550	35	68	3	656
1981	469	29	78	2	578
1982	160	12	92	5	269
1983	95	3	18	1	117
1984	77	5	3	2	87
1985	56	3	3	6	68
1986	40	2	5	8	55
Total	10,740	957	2,198	48	13,943

Sources: Dr. Nestór J. Juan. Jefe, Departamento de Zoonosis, Reservorios y Vectores.
Silvia Beatriz Cozzo. Asesora Médica del Departamento de Zoonosis Reservorios y Vectores, Ministerio de Salud y Acción Social.
VERA (Centro Panamericano de Zoonosis - OPS/OMS), vol. XII, nos. 7-12, 1986.
Honigman, M.N. (51).

Table 14. Cases of Rabies in Humans in Argentina, 1976-86

Year	1976	1977	1978	1979	1980	1981	1982	1983	1984	1985	1986
No. of Cases	19	8	9	6[a]	0	3	1	1	2[b]	0	0

[a] 1 case caused by a cat bite.

[b] 1 case caused by a bat bite.

Sources: As in Table 13.

Vampire Bat Rabies

Rabies in vampire bats is not as directly related to human health as dog rabies and is thus reported with less concern. Its importance can, however, be estimated by the total number of bovine cases reported annually, the percentage of animals infected in a given area, and the animal (and human) mortality in outbreaks.

Table 15. Bovine Paralytic Rabies in the Americas

Country	Number of cases/year	Ann. mortality (estimated)	No. of cattle vaccinated/year	Annual loss (US currency equiv.)
Argentina	18,000 (1964)	50,000	100,000 (1965)	$10,000,000 (1964)
Brazil	32,200 (1965)	200.000	1,300,000 (1965)	22,000,000 (1965-66)
Bolivia	20,000 (1965)	50,000	5,000 (1965)	1,500,000 (1965)
Br. Honduras	815 (1962)	2,000	200 (1962)	100,000 (1961)
Costa Rica	132 (1964)	10,000	18,000 (1963)	365,000 (1962)
Colombia	5,300 (1964)	50,000	150,000 (1963)	1,260,000 (1964)
Ecuador	930 (1962)	5,000	4,500 (1962)	850,000 (1963)
El Salvador	1,080 (1961)	3,000	7,000 (1964)	108,000 (1961)
French Guiana	600 (1958)	1,000	-[a]	60,000 (1958)
Guatemala	1,120 (1964)	12,000	8,000 (1964)	168,000 (1964)
Guyana	2,000 (1957)	3,000	30,000 (1963)	43,000 (1959)
Honduras	348 (1960)	6,000	5,000 (1963)	87,000 (1960)
Mexico	1,502 (1963)	90,000	1,000,000 (1963)	10,400,000 (1964)
Nicaragua	831 (1962)	10,000	8,000 (1964)	200,000 (1962)
Panama	218 (1962)	8,000	5,000 (1963)	115,000 (1962)
Paraguay	320 (1963)	5,000	2,000 (1964)	94,000 (1963)
Surinam	733 (1963)	2,000	5,013 (1963)	55,000 (1963)
Trinidad	2 (1965)	500	24,047 (1963)	5,000 (1961)
Uruguay	83 (1965)	2,000	-	63,000 (1965)
Venezuela	215 (1965)	5,000	53,032 (1963)	119,000 (1960)
Total	86,439	514,500	2,724,792	$47,592,000

[a] No information available.

Source: Acha (61).

The difficulty in estimating the number of annual cases is shown by the wide range of estimates made. In Mexico, for instance, the annual bovine mortality has been variously estimated at 10,000 (58), 50,000 (59), or 100,000 head (60). Acha (61), in 1967, estimated a hemisphere mortality of 514,500 yearly, at a cost of US$47,592,000. (Table 15). The numbers reported emphasize the great disparity between those cases actually reported and the estimated "real" annual number; the reported cases range from 3-60% of the estimated annual death toll.

In view of the massive bovine mortality, the lack of official reporting is at times astounding. In 1970 a one-paragraph mimeograph report appeared on an outbreak of vampire paralytic rabies in the Cercado de Tarija Valley of southern Bolivia, involving 260,000 head of cattle between 1954 and 1955 (CEPANZO) (62); it then spread to an additional 4 northern Argentine provinces between 1960 and 1968 (63)

Table 16. Clinically Normal Cattle with Rabies Virus in Brain, Killed at Ferreria Slaughterhouse (Mexico City), by State of Origin, 1970

State	Number of cattle examined	Number of cattle positive	% of cattle positive
Vera Cruz	525	12	2.3
México	157	9	5.7
Oaxaca	85	11	12.9
Tabasco	98	2	2.0
Michoacán	125	4	3.2
Yucatán	10	2	20.0
Total	1,000	40	4.0

Source: Martell and del Valle (65).

where at least 5,000 more cattle died. In some of these areas no cattle were left. It is important to note that no official reports of this massive outbreak appears to have reached national or international reporting agencies.

Another example of the limited reporting of bovine cases was a smaller outbreak that occurred in Tuxtepec, Oaxaca, Mexico, between May, 1967 and July, 1968, where only 1 laboratory diagnosis was made but a total of 571 animals actually died (64). Still further evidence of the toll exacted by rabid vampires is the startling percentage of rabid cattle found among those sent to slaughter. In 1971, Martell and del Valle (65) reported that 40 of 1000 (4%) randomly selected (and apparently healthy) cattle about to be killed in the Mexico City slaughterhouse were shown by immunofluorescence staining and mouse inoculation of brain materials to be infected with rabies (Table 16).

The hundreds of thousands of doses of rabies vaccine sold for vaccination of cattle give additional evidence of the importance and geographic distribution of the disease, even in areas where no bovine rabies has been reported.

The epizootiology of vampire paralytic rabies is mostly known through studies of outbreaks, and little attention has been paid to the enzootics which follow. Outbreaks begin with an increase in both rabid vampire bats and in bat bites. In one outbreak in Trinidad 14.3% of the

vampire bats examined were found to be rabid (66), yet in enzootic areas the rates varied from 0.46% (67) to 1.6% (68).

A better indicator of infection is the prevalence of serum neutralizing antibodies which result from bat-to-bat contact. This sometimes reaches 36% after epizootics (69). A detailed serological study of vampire bats during a bovine out break was done by Lord *et al.* (70) in the area of northern Argentina. Vampire bats were bled prior to, during, and after the outbreak, and the percentage with rabies antibodies rose from 3.1% to 6.6% to 16.8% although no virus was detected in any of the 1,024 bats examined during those 3 periods. The authors conclude that "It appears that, on entering a vampire bat population, rabies virus successfully infects much of it, possibly killing some individuals and causing immunization of others. Upon removal of sufficient susceptibles in these ways the epizootic presumably subsides or moves on to the neighboring populations."

Mongoose Rabies

Mongooses were imported into various Caribbean islands in the 1860s, and mongoose rabies has been known in Grenada, Puerto Rico, Cuba, the Dominican Republic, and Haiti for several decades (71). The numbers of rabid mongooses always make up a significant percentage of all wildlife rabies cases there, as well as the cause of many human post-exposure vaccinations and human deaths.

The studies carried out by Everard *et al.* in Grenada show that the epizootiology of mongoose rabies differed in different parts of that small island (72). In spite of those differences in the infection rate, many mongoose sera examined (in all parts of the island) were found to contain serum neutralizing (SN) antibodies, indicating that rabid mongooses readily bite other mongooses and that these animals are quite resistant to rabies, since many develop antibodies instead of succumbing to the virus. There was a direct inverse relationship between the rabies infection rate and the percentage of animals having rabies antibody: the first year (1971) mongoose populations were examined, the highest rabies infection rate coincided with the lowest antibody rate, while during the last year (1974) the highest antibody rate was found along with the lowest rabies infection rate. The authors concluded (72) that: "...The relationship between rabies and rabies antibody in

Table 17. Reaction Patterns of Viral Isolates from Rabies Enzootic Areas of Central and South America

Major host/ area/ year	Hybridoma number[2]																	# of isolates
	1	2	3	7	10	11	12	15	16	17	18	19	T	P	5	8	13	
Dog/ Argentina 1979-1980	+	+	+	+	+	+	+	+	+	+	ø	+	+	ø	+	+	+	7
Mexico 1978																		6
Dominican Republic 1987																		2
Guatemala 1984																		1
U.S.A. (Mexican border area) 1985-86																		7
Desmodus rotundus/ Brazil 1986	ø	+	+	+	+	+	+	ø	ø	+	ø	+	+	ø	+	+	+	1
Venezuela 1978																		1
Argentina 1984																		1
Tadarida brasiliensis/ Chile 1985-86	ø	+	+	+	+	+	+	ø	ø	+	ø	ø	+	ø	+	+	+	9

For an explanation of symbols, see legend to Table 3.

Grenada mongooses was determined for a population of 1.0 to 2.5 per acre under small-island conditions... In the present study the SN antibody prevalence rate increased from 20.8% in 1971, when 3.5% of the population was rabid, to a rate of 43.2% in 1974 when only 0.6% was rabid. Extrapolation of the linear regression line equation suggests that almost 60% of mongooses would have rabies neutralizing antibody at the end of an epizootic before transmission ceased and no rabies was present in the population... Since rabies virus appears not to be very

pathogenic for mongooses, more than 40% of a mongoose population can acquire antibody by natural exposure in the field."

Rabies in Other Species

Occasional cases of rabies have been reported in many other wildlife species of animals, including foxes, skunks, coyotes, etc., but these have not made up a significant part of the rabies cases in any Latin American country at any time. As canine rabies and vampire paralytic rabies are controlled in the hemisphere, the proportion of rabid wild animals can be expected to increase, as it has in the United States and Canada since the 1940s (1).

Mab-N Analysis of Rabies Isolates from Latin America

Although there has been no systematic analysis of antigenic variation among rabies virus isolates from enzootic areas of Central and South America, the few samples which have been tested suggest that a compartmentalization of rabies variants to a particular host species similar to that observed in North America may also occur here. Table 17 summarizes the results of Mab-N tests of 42 rabies isolates. The largest group comprises 26 isolates from dogs or from infections believed to have resulted from contact with rabid dogs. This reactivity pattern is found in isolates collected in areas of active dog rabies extending from Argentina to the Mexico - United States border and in 3 human rabies cases which, although diagnosed in the United States, were a consequence of rabid dog bites in Mexico. Different reactivity patterns characterized isolates from 9 Brazilian free-tail bats collected in Chile and 3 vampire bats (*Desmodus rotundus*) collected in Argentina, Brazil and Venezuela, allowing viruses infecting these species to be distinguished from each other and from the rabies variant found in dog rabies enzootic areas. Two virus isolates from domestic animals (dog, Belize, 1987; cow, French Guinea, 1986) were identical to variants found in rabies-infected vampire bats and 1 isolate from a rabid cat in Chile in 1985 was identical to Brazilian freetail bat isolates in Chile. Four isolates (2 dogs and 1 cow, Argentina, 1980; mountain lion, Mexico, 1987) could not be grouped by reactivity pattern.

CONCLUSION

It is clear that much progress has been made in understanding the behavior and movement of rabies virus in nature. Characterization of isolates with monoclonal antibodies has identified distinctive antigenic differences that can be used as an epidemiologic marker system for the study of strain prevalence and distribution. Further progress, however, will depend on identifying those factors that permit the virus to become selectively entrenched in an ecologic niche.

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PERSISTENCE OF RABIES IN WILDLIFE

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INTRODUCTION

Rabies has been reported for at least 2,000 years (1; Wilkinson, this volume). It occurs on all the continental land masses except for Australia and Antarctica (2). It affects a wide variety of mammals including the carnivores, ungulates, rodents and bats. The canids are most commonly involved both as victims and vectors. Rabies has killed humans throughout its history but today, especially in western countries, the disease is more of a threat than a killer. Nevertheless it can be a significant economic problem. In Ontario, for instance, rabies costs over $20 million per year from compensation costs, pet vaccinations and the yearly treatment of over 2,000 exposed persons (3).

Rabies has waxed and waned throughout its history in Europe and North America. The current epizootic in Europe began in Poland in 1939 and has swept westward at a pace of 20-60 km per year (4; see also Blancou, this volume). The major vector has been the red fox (*Vulpes vulpes*). In North America, wildlife played a minor role in rabies during the first half of this century. Most cases occurred in dogs in small isolated outbreaks. Since the 1940s, however, wildlife rabies has been dominant over wide areas. In Canada, rabies swept south from the arctic into the coyote (*Canis latrans*) population in Alberta and the fox populations of Manitoba and eastern Canada. It quickly died out in the canid populations of the prairies but remained in the east predominantly in southern Ontario. A few years later the prairie provinces were invaded again by an epizootic in striped skunks (*Mephitis*

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mephitis) originating in the midwest United States. Raccoon (*Procyon lotor*) rabies has been expanding along the eastern seaboard from Florida since the 1950s (5). It is currently moving northeast through the ridge and valley country in Pennsylvania.

Thus, rabies is a successful disease. It has persisted over time and space achieving a worldwide distribution in a variety of species. The reasons for this success are unclear. However, given the growing interest in rabies control, it is important to explore the mechanisms by which rabies persists. The purpose of this chapter is to describe and discuss possible mechanisms. As well, this chapter will examine reasons for the current wildlife rabies enzootic in southern Ontario.

PERSPECTIVES ON PERSISTENCE

There are 3 general components in the ecology of any disease: the pathogen, the host and the environment (physical and behavioral) in which the host and pathogen interact (6). Furthermore, the peculiar patterns of disease in any given situation can only be completely understood by examining all 3 components jointly. Since most infectious diseases are not distributed uniformly in time or space, we may conclude (although there is no real proof) that only certain combinations of those components are favorable to the existence of a given disease. Furthermore, since populations and environments are dynamic then the continued existence of a disease must be due either to: (i) the pathogen's ability to develop new strains to suit new conditions; (ii) variability in the pathogen's properties that allow it to take advantage of a wide range of conditions; or (iii) changes in the host and/or the environment that create a continuous set of new opportunities that the pathogen can exploit without having to change itself.

The annual worldwide waves of new strains of influenza are a good example of (i) above. As well, influenza is also a good example of (iii) since the high volume of the international movement of people makes it possible for the new strains to become pandemic. Paradoxically, the rapid worldwide movement of influenza requires that the virus change rapidly to survive since the exposure (and subsequent immunity) of many persons to one year's strain will protect the population against future epidemics. Foot-and-mouth disease is a good

example of (ii) since it can spread in many ways: by airborne droplet nuclei over long distances; by direct contact between animals; and by fomites (it can survive for long periods outside the host) including transfer on hay and passaging through the gastro-intestinal tract of birds migrating over long distances (7).

In the case of rabies, there is some evidence that all 3 of those possibilities are correct. Thus, a reasonable strategy for examining the persistence of rabies is to examine each possibility in turn. That is the purpose of the next sections.

Changes in the Rabies Virus.

Before this decade, most of the literature held that there was a single rabies virus. Differences in incidence between populations were attributed to differences in susceptibility of species. Rabies vaccines appeared to provide protection against all rabies and all test specimens from different areas reacted similarly to the standard immunological tests (8).

However, since the development of monoclonal antibody techniques (9,10), research has shown that clearly different strains exist between certain populations (11-13; see also Smith and Baer, this volume). Furthermore, the variation seems to be geographic. For instance foxes, skunks, raccoons and domestic animals from the eastern arctic through to New York show the same strain whereas skunks, raccoons, horses and cattle from Manitoba have a different strain (13). In southeastern U.S., raccoons, foxes, cattle and woodchucks share the same strain which is again different from other strains. As well, samples in that area showed no difference between 1978 and 1983 (12) so that those strains appear stable over the short term.

This evidence matches the patterns of incidence in North America described above; i.e., enzootic fox rabies in the east centered on southern Ontario, enzootic skunk rabies in the mid-western U.S. and the Canadian prairies, and epizootic raccoon rabies spread north along the eastern seaboard of the U.S. Therefore, some unique combinations of virus, host and environment must exist to maintain those distinct geographic clusters of rabies. For instance, the arctic fox virus that spread south in North America in the 1950s probably caused the fox rabies epizootics from Manitoba eastwards and the coyote rabies

epizootic in Alberta. Rabies then died out everywhere but southern Ontario. A subsequent invasion of skunk rabies from the American Midwest has remained in the prairies to this day. In sum, the geographic variations in strain and incidence suggest that persistence does not depend on a rapid change in the rabies virus. Variation in the virus must exist but only to allow rabies to take advantage of different and/or changing host/environment combinations. The question then becomes how different strains of rabies manage to survive until an opportunity for spread occurs.

Part of the answer may be a reservoir species. In North America bats are the likely candidate. Unlike the other species, bats harbor a variety of strains. Koprowski *et al.* (14) isolated 16 rabies strains from bats in Maryland and Pennsylvania and Smith *et al.* (12) found 4 different strains from 7 specimens in Maryland. Other factors enhance the notion of bats as reservoirs. First, bats occur everywhere in North America and several species migrate overlong distances. Second, transmission from bats to other animals can now be identified and that route has been verified (8). Third, on the basis of available incidence data, rabies transmission from bats to other species is rare. Finally, the reported incidence of rabies in bats is low even in the Pacific west where bats are the major species diagnosed with rabies. Thus bats may be an ideal reservoir for rabies. They are widespread, mobile, harbor many strains and are relatively unaffected themselves. Proving this suggestion depends on further monoclonal studies to demonstrate that rabies strains in bats can cause epizootics in other species.

There is no clear evidence for reservoirs in other parts of the world although different rabies strains do exist in many species. Bat rabies is found in most places but there is no evidence as yet that those bats harbor several strains of virus. Interestingly, the Duvenhage virus, a rabies-related virus originally found in Africa, has been found in bats in northern Germany and other European countries (15; see also King and Crick, this volume). This suggests that long range migration of bats can set up reservoirs of rabies. Fortunately, there is no evidence that this bat strain has been transmitted to other wildlife in Europe, but there have been several human deaths from rabies (or rabies-related virus, serotype 4) following bat bites (16).

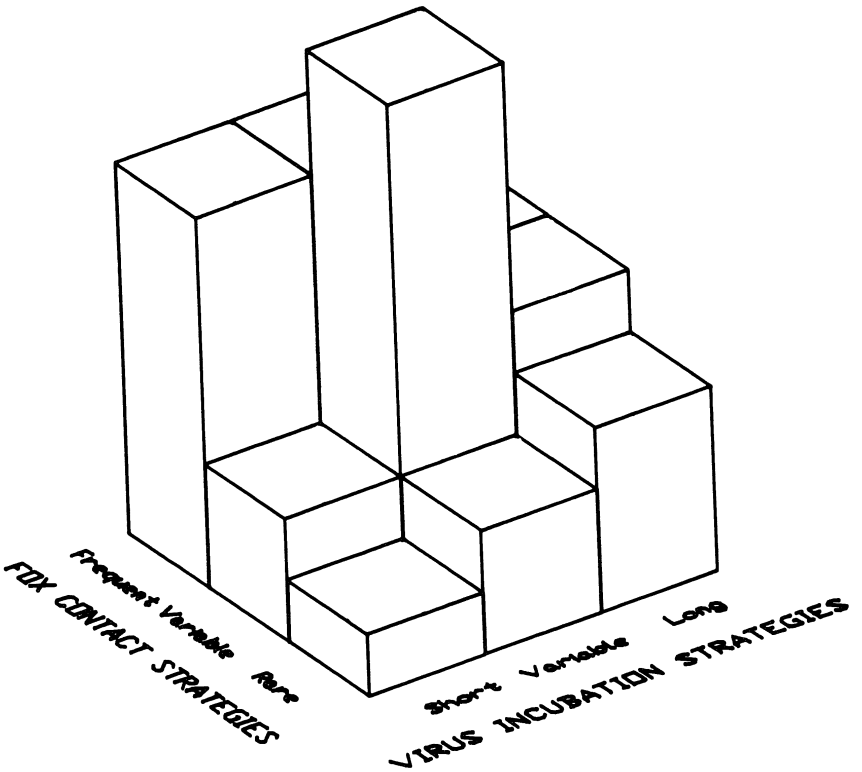

Figure 1. Shows the hypothesized interplay between fox contacts and the variability of the incubation period as a game theory matrix. The heights of the boxes represent the payoffs to the virus, in numbers of infected foxes, from each combination of fox and virus strategy. Modified from Bacon (19).

Another possibility for a reservoir is that an animal becomes immune after infection but continues to shed virus. In the fox rabies enzootic area of Ontario about 3% of non-rabid foxes show rabies antibody (17). In Switzerland, Steck (18) found that less than 2% of 239 foxes survived and became immune after experimental infection. Given that fox populations have high turnover rates and given that no-one has demonstrated virus shedding from immunized animals, this mechanism is not a factor in the persistence of rabies. In sum, questions of virus changes, rabies reservoirs and transmission from those reservoirs are unresolved and will remain unresolved until many more monoclonal studies are completed. However, as the next sections

illustrate, there are other plausible explanations of persistence that account for the recent history of sylvatic outbreaks in Europe and North America.

Properties of the Virus

Bacon (19) has examined the properties of the rabies virus from a game theoretic perspective. Here the virus plays a survival game against its environment, the fox society. This is a useful perspective for discussing how the properties of the rabies virus may affect survival. For instance, the incubation time of rabies is extremely variable, ranging from 10-14 days to many months (20) with a mode of about 3 weeks (21). In one of Bacon's examples, the virus plays the game by changing the length of its incubation period while the foxes change their movement strategies (Fig. 1). The optimum survival strategy for individual foxes is to make rare contacts. However, the need for breeding and raising young means that foxes must make more frequent contact although the level of contact will vary by season. Thus, foxes accept a variable contacts strategy which, in turn, encourages the virus to adopt a variable incubation period strategy for maximum payoff. Short incubation periods mean rapid spread when conditions are right and long incubation periods hold the virus until appropriate contacts are made. A concentration on short incubation periods would cause an epizootic to burn out, while a concentration on long incubation periods might prevent an epizootic from igniting in the first place.

Bacon also uses a game theoretic approach to argue that the range of symptoms induced in a rabid animal is a sophisticated adaptation for ensuring persistence. Rabid animals may exhibit 'passive' or 'furious' behavior, or both, before the final paralytic or 'dumb' stage. During the passive period the animal sheds virus, is friendly and continues to interact with its unknowing family. During the furious stage the fox seeks out and attacks other animals. Pure passive behavior would increase the potential for rapid spread and burnout while a pure furious strategy would encourage the evolution of avoidance behaviors by the fox population. Thus, the virus should encourage both behaviors. Obviously the best strategy for foxes to counter any virus strategy would be to live solitary lives. Under those conditions the virus would

have to adopt a furious strategy. However, fox reproduction requires some social contact. Thus, the virus will gain the most by inducing a range of behavior in the host population.

Variability in virulence and the amount of virus secreted in saliva may also help to maintain rabies in a host species (5). However, there is no evidence for those types of variation. Furthermore, it does not really matter whether the variability in incubation period or symptomatic behavior or virulence or excretion is due to the virus itself or whether that variability is due to the interaction between host population and the virus. The end result is the same, despite the mechanism.

The current evidence favors the interaction hypothesis. For instance, it is known that the length of the incubation period is affected by the size of the initial dose and the site of the infection (22,23). Those conditions depend on the nature of the contact between animals which is influenced by whether the infecting fox is in the passive or the furious phase of rabies. The length of the incubation period has also been linked to stress. An infected fox may not show symptoms until a period of high stress such as dispersal, mating or parturition. Coincidentally, those periods are also times of highest contact between foxes.

Properties of the Host and the Environment

The previous sections have argued that, over the short term of the current rabies outbreaks (about 40 years), the interaction between the virus and the host population has promoted the survival of the disease. The virus has not changed in any significant way and has taken advantage of situations suited to the maintenance of rabies. The purpose of this section is to discuss those features of the host population and the environment that promote the survival of rabies. The relevant features of the host population are: (a) life cycle; (b) territoriality; (c) density; (d) movement behaviors; and (e) reproduction. The relevant features of the environment are: (a) the suitability of the habitat; (b) the heterogeneity of the habitat; (c) the size of an infected area; and (d) the existence of man-made and physiographic barriers.

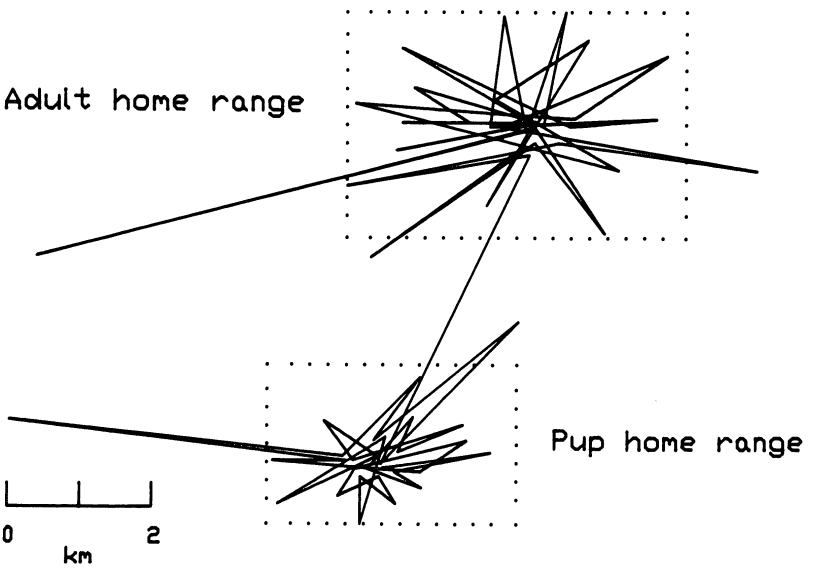

Figure 2. Typical fox dispersal pattern obtained from radio tracking. The end points of each line are the locations of successive nightly fixes taken on a radio-collared fox from May to December. The pattern shows the pup home range in late spring and summer and then the final home range in late fall and early winter after dispersal in late October. Note the occasional long 'exploratory' movements from each home range.

The Host Population

In the fox rabies areas of Europe and North America, foxes are born in March and April and remain close to the natal den until late spring. During the summer the pups explore an ever widening area focused on the natal den and within the home range defined by the parents' movements. In the late summer and early fall, the juveniles (about 6-7 months old) begin to explore adjacent territories and then usually make a permanent dispersal to a new territory by late fall or early winter. In Ontario, those dispersal movements have a negative exponential distribution with a mean of 26.1 km for males and 7.1 km for females (24). Fig. 2 shows a representative dispersal pattern for a male juvenile. The majority of movements are short but occasionally dispersals occur over distances of up to 200 km (25).

In the enzootic areas home ranges vary between 1-8 km^2 (26) and densities vary between 1 and 2 animals per km^2 (27). The home range is not uniform since the foxes tend to use some portions more frequently. There is some evidence of overlap between territories. During dispersal and mating, foxes explore nearby territories presumably to find empty territories or ones with potential mates. This 'spacing' behavior continues throughout the year amongst adults, and territories vacated by the death or movement of an occupant can be filled in from adjacent higher density territories. Macdonald has termed this the 'vacuum effect' and argues that it is a major reason why the 'kill' strategies have failed to control rabies in Europe (28). Thus, by reducing density, the vacuum effect may protect certain areas from rabies. The protected areas serve as population reservoirs to ensure a continued supply of susceptibles.

Reproduction in the fox rabies enzootic areas is also quite high. In Ontario, for example, mean litter size for adult vixens ranges between 6-8 (20). This is higher than anywhere else in the world. Throughout Europe, mean litter sizes average 4.3 pups (29). The reason for the differences between the 2 areas is not clear although North American foxes live at lower overall densities and have more exclusive and larger territories. Presumably each territory has a higher resource base and, consequently, a higher reproductive capability.

In sum, the structure of fox populations in the rabies enzootic areas of North America and Europe is conducive to rabies in several ways: (i) the dispersal and spacing behaviors ensure spread; (ii) territoriality coupled with the seasonally biased higher contact rates limits spread and slows down the progression of the disease; and (iii) the high reproductive capability and the spacing behavior means that areas hit by rabies will be repopulated in a relatively short time, thus creating a new population of susceptibles.

The Environment

The persistence of fox rabies in Europe and North America means that, overall, the habitat must be suitable for foxes. Unfortunately it is difficult to measure habitat as there is no good method for measuring absolute densities of foxes. However, if we make the assumption

that high rabies incidence reflects high population densities then it is possible to make some judgements about habitat. First, good habitat is 'patchy'. The good patches have some or all of the following elements: (i) a mix of forest and field; (ii) good natural drainage (usually associated with hilly terrain); (iii) pasture rather than cash crops; (iv) smaller fields producing a large number of 'edges' in the environment (30); and (vi) soils or geological structures suitable for denning. The poor patches are flat and have poor natural drainage. They also tend to have large fields that limit the number of 'edges' and restrict the number of potential denning sites.

The heterogeneity or patchiness of the environment can influence persistence in a variety of a ways. Sayers *et al.* (31) note that high rabies density in France correlates with greater diversity of vegetation while low density areas have more uniform land cover. Obviously an area with poor habitat will not sustain rabies. An area with uniformly good habitat might ignite too quickly and burn itself out. There must be a balance of good and poor habitat to maintain spread but slow it down sufficiently to allow populations to rebuild behind the epizootic. Areas of poor habitat may provide the 'traps' in the system to slow down spread. Heterogeneity would allow isolated pockets of animals to escape the virus and provide the breeding stock for the next generation of susceptibles.

In addition to the influence of heterogeneity, we should also expect that the size of the area under consideration (as determined by physiographic barriers), should have an impact on persistence. Despite good habitat, a small area might not have the reproductive capacity to be able to supply a sufficient number of susceptibles in time to allow rabies to be self sustaining. Hence there must be some minimum area (threshold) for rabies to persist given the particular combination of land cover and reproductive capability of the area. Failing that there must be some other spread mechanism to allow areas below that threshold to be reinfected from time to time. Some possibilities, such as reservoirs, very long incubation periods and long range dispersals have already been discussed.

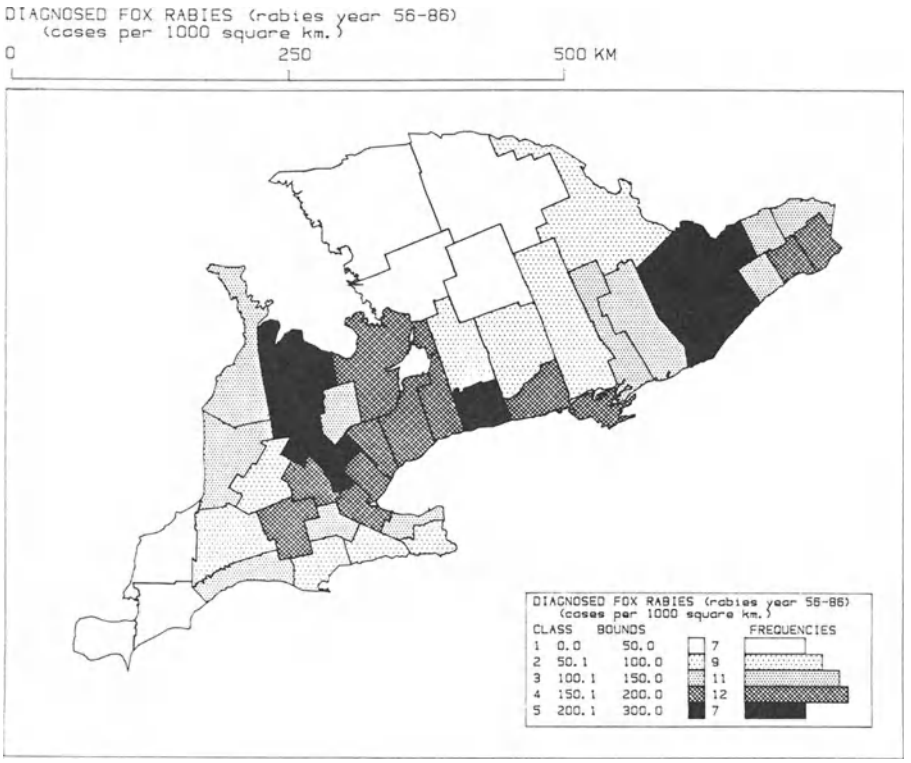

Figure 3. Distribution of all reported fox cases in southern Ontario by county by 'rabies year'. Since incidence peaks in the fall and winter a 'rabies year' is taken from July 1st of one year to June 30th of the next calendar year. Hence 1986 means July 1/86 to June 30/87.

PERSISTENCE IN ONTARIO

Fox rabies invaded southern Ontario in the mid 1950s as part of the great epizootic that swept down from the arctic and across eastern North America to the Maritimes and northern New York. It has died out everywhere but southern Ontario and small adjoining areas along the St. Lawrence River in northern New York and southwestern Quebec. Why? Which of the factors or combinations of factors discussed above have allowed rabies to persist in southern Ontario?

Southern Ontario (Fig. 3) is a good location for examining persistence for several reasons. First, although the case records generally deal only with occurrences involving human contact, they have

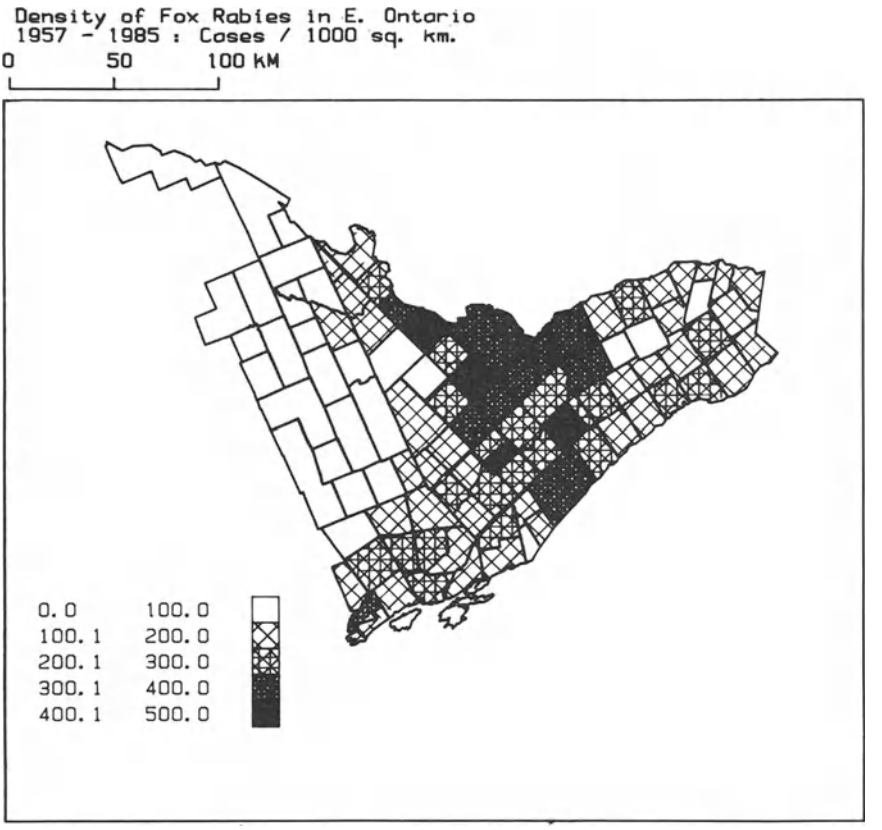

Figure 4. Distribution of reported fox cases in eastern Ontario plotted by township. Township data was not available after 1985. Cases are concentrated in the 'core' area between Ottawa (on the north) and Brockville (on the south). The low incidence area to the west is the Canadian Shield and the low incidence area in the east is generally flat and poorly drained. The Ottawa River is the northern boundary of the unit and the St. Lawrence River is the southern boundary.

been collected uniformly over the entire enzootic period. In Canada, responsibility for rabies reporting lies with federal veterinarians assigned to local districts who report following standard federal guidelines. We have not found any evidence of a reporting bias either by reporting district or by human population density (21,32). Although the reported number of cases is probably much less than the actual number of cases (2-10% in Europe (33)), we feel that those reports are reasonable indicators of the true patterns of incidence. Second, for most of the year, southern Ontario is an isolated self-sustaining

'island' of enzootic rabies. It is surrounded to the south, east and west by the Great Lakes and the Ottawa and St. Lawrence Rivers, and is bordered on the north by the Canadian Shield. The 'island' is bridged by ice along the Ottawa and St. Lawrence rivers from January to March. However, we have examined patterns of incidence on both sides of those rivers for the past 10 years and have not found any evidence of spread over the ice from either Quebec or New York. Indeed, the evidence suggests that spread from Ontario has been responsible for the cases in Quebec and northern New York. The Canadian Shield provides a wide border of poor habitat. Incidence is very low. The area is primarily boreal forest set on rough and rocky terrain dotted with many small lakes and streams.

Finally, over the past several years we have built and refined a fairly complex simulation model of a fox population with rabies (24). In as far as possible we derived the parameters and behavioral rules for the model from field studies of fox populations in Ontario. As well, we performed an extensive series of experiments to validate the model; i.e., to make sure that its temporal and spatial output matched the general temporal and spatial features of rabies in southern Ontario. A major component of the validation process was to understand the conditions under which the model would allow rabies to persist.

Factors Affecting Persistence in Southern Ontario

The analysis of rabies incidence patterns in southern Ontario, experience gained in developing the Ontario Rabies Model and the research discussed in the previous sections suggests that the following factors affect the persistence of rabies in southern Ontario:

(i) the existence of rabies units;
(ii) the size of the rabies units;
(iii) heterogeneity in habitat;
(iv) reproduction and mortality;
(v) incubation period;
(vi) density and contact rate.

Consider, first, factors (i) and (ii) that deal with regions. Fox rabies in southern Ontario is distinctly clustered (Fig. 3). Major clusters occur in eastern Ontario, central Ontario along the north shore of Lake Ontario, a loop of counties around Metropolitan Toronto,

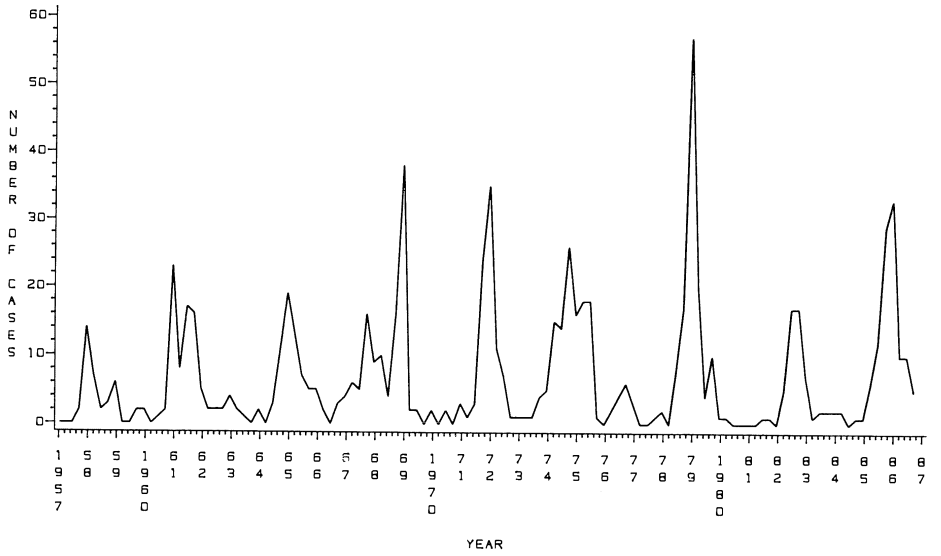

Figure 5. Fox rabies incidence by quarter in Renfrew County. The southeastern part of the county is part of the high incidence 'core' in eastern Ontario. Note the strong cycle of incidence with a 3-4 year period.

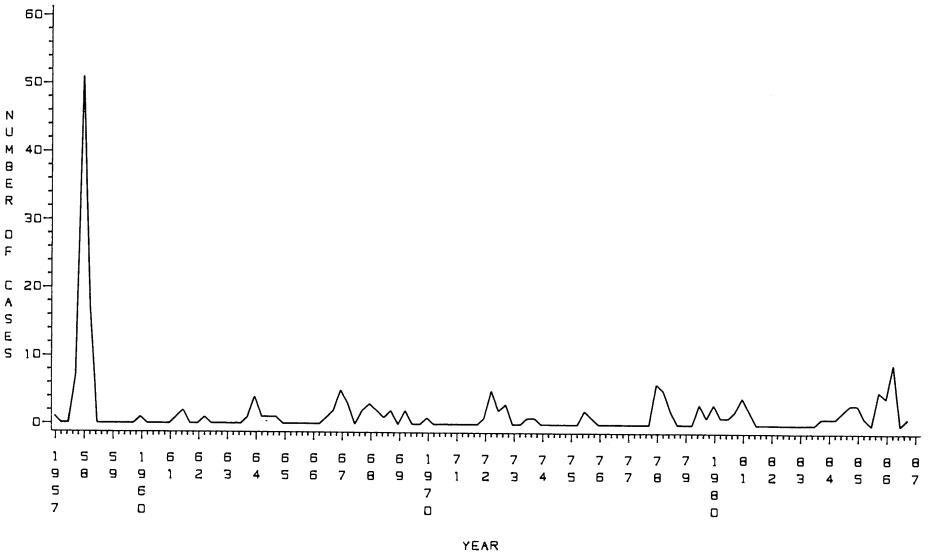

Figure 6. Fox rabies incidence by quarter in Prescott County. This county is one of the 5 counties in the flat and poorly drained low incidence area in the eastern tip of eastern Ontario. Note the weak cycle and irregular period. The large peak in 1958 was the start of the current enzootic.

counties to the immediate south of Georgian Bay and counties in an area centered on Oxford County. We grouped counties with similar time series and incidence and found 10-12 clusters. Temporal patterns are distinctive for each cluster and are out of phase with their neighbors (26). We have termed those separate clusters 'rabies units'.

A good example of a such a unit is eastern Ontario (Fig. 4). This unit is isolated from all other areas by the Canadian Shield on its western and northern flanks and by the Ottawa and St. Lawrence Rivers to the northeast and south. The spatial and temporal patterns of incidence within that unit have been very consistent over time. Incidence is concentrated in the 5 county core of the unit and has a strong 3 year cycle (Fig. 5). Incidence in the area immediately adjacent to the core has a weak cycle that tends to be out of phase with the core and with the other peripheral counties (Fig. 6).

More important, the core area in eastern Ontario is about 5,000 km^2. In our experiments with the Ontario Rabies Model, we found that we could not simulate an enzootic situation consistently unless the area being modelled was at least 4,000 km^2. MacInnes (8) provides some support for this conclusion. He notes that when fox rabies originally spread from the north into Ontario across the Canadian Shield it passed through the Clay Belt agricultural area between Cobalt and Kirkland Lake but did not persist. The area is about 3,000 km^2.

Southern Ontario has 5 units that meet the threshold requirement of 4,000 km^2 for persistence. This suggests an additional mechanism for persistence. Long range dispersal of rabid animals between those areas is possible. As well, our work with the Ontario Rabies Model suggests that low levels of introduction will trigger new outbreaks. Thus if even rabies were to die out within a unit there is a possibility for subsequent reinfection from another unit. In a sense, this is the heterogeneity argument at the macroscale. It may also prove to be the primary mechanism for maintaining rabies in southern Ontario. If this is correct, then control measures should be concentrated on those units with the objective of breaking the chain of infection. Furthermore, given its relative isolation, the eastern Ontario unit would be a logical place to test control measures.

We have also studied the relationship between rabies incidence and habitat in the eastern Ontario unit. High incidence is associated with areas of limestone, good drainage and agricultural practices associated with small farms and sheep (34). The regression model in this study explained about 50% of the variance in the data. The unit used in this study was the township, an administrative area averaging about 100-200 km^2. That unit is, perhaps, too large since it masks variation at the scale of a fox home range (2-4 km^2). However, the result was significant ($p = .001$) and did indicate a strong relationship between rabies incidence and habitat at the township scale. Sayers *et al.* (31) also report that limestone and diversity of vegetation are associated with high case density and persistence of rabies. This work complements, but does not prove, the argument that habitat variations support persistence. Macdonald and Voigt (35) list some of the problems with the "habitat-fox-rabies" approach. We are preparing a series of simulation runs with the Ontario Rabies Model to test the impact of habitat variations on persistence.

Ontario vixens have a high reproductive capacity. The mean value of 8.2 pups/litter reported for Ontario is the highest in the world. This suggests that the fox population is responding to a high mortality pressure. Given that the level of reproduction is near the physiological limits for foxes, the fox population is probably not capable of a significantly increased reproductive response should mortality pressure increase. That being so, a high reproductive capacity is a necessary but not sufficient mechanism for maintaining rabies in Ontario. There must also be a mechanism for keeping total mortality in check if rabies is to be maintained.

We have demonstrated that such a mechanism must exist by studying the interaction between the fur harvest (trapping) and rabies the 2 major killers of foxes in Ontario (26). We discovered that, over a 4 year period (to average out the cyclical effects of rabies), the product of harvest and rabies mortality was a constant. Those mortalities were compensatory such that a high harvest over 4 years meant low rabies and vice versa. On a year-to-year basis the results were complex and depended on the timing of the harvest relative to the arrival of rabies in an area. We have a mechanism in the Ontario Rabies Model to

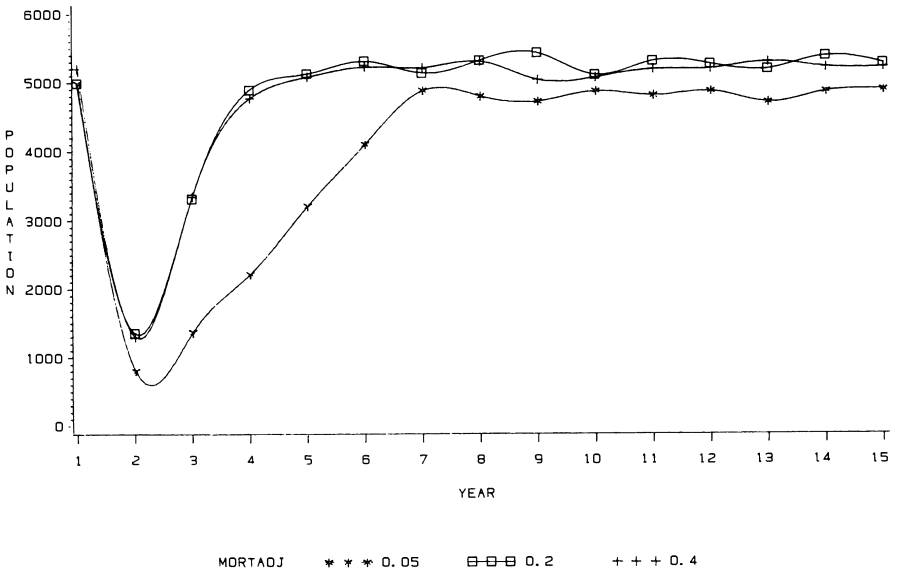

Figure 7. Results of population recovery experiments with a low mortality adjustment mechanism (0.05) and with higher levels (0.2 - 0.4). Non-rabies mortality is lowered as the adjuster increases. Note that the results indicate a ceiling on recovery; i.e., we could not force the population to recover faster than 3 years without increasing reproductive capacity well above that observed in Ontario.

seasonally adjust non-rabies mortality to compensate for rabies deaths; i.e., when populations are forced low by rabies, non-rabies mortality decreases and the remaining foxes survive longer (presumably by having less competition for food). When this mortality feedback mechanism was at low levels or turned off, fox populations in the model would not recover fast enough to sustain the 3-4 cycle observed in Ontario (Fig. 7). Furthermore, rabies would not persist without that mechanism. Work with the model also demonstrated the impact of incubation period on persistence. The rabies units in Ontario, described above, exhibit cyclical incidence patterns, with a period of 3-4 years. The length of this cycle reflects the rate of spread of rabies relative to the ability of the population to recover. As well, areas with longer cycles or irregular cycles are areas in which rabies does not always persist. Thus 3-4 year cycles also seem to be a characteristic of persistence in southern Ontario. Incubation period had a marked impact on the length

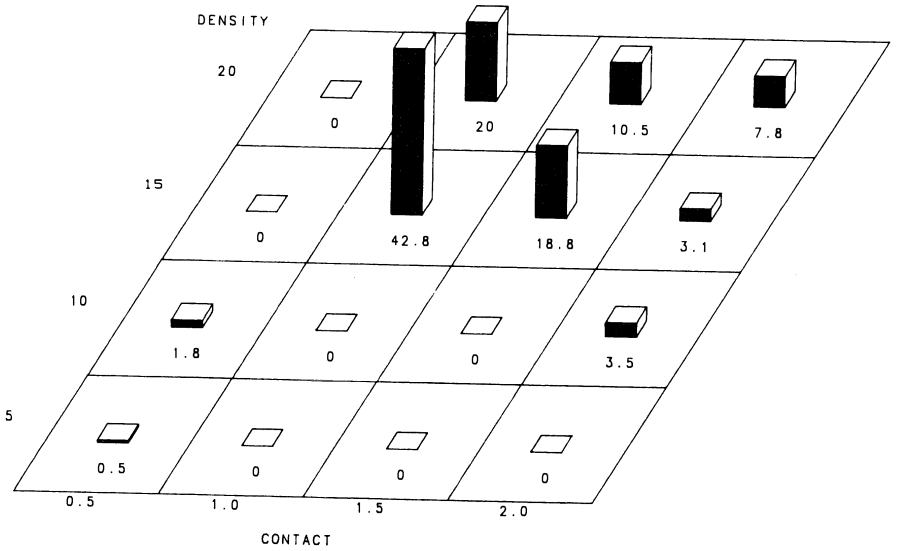

Figure 8. Results of an experiment examining the joint effects of density and contact rate. The density values are given in foxes per 10 km^2. Hence a value of 20 means 2 foxes per km^2. The contact rates are relative. Since we cannot measure contact in the field we can only adjust contact rates relative to other model runs. The height of the bar is proportional to the percent of model runs in which rabies persisted for a 25 year period in an area of 4,000 km^2. A contact rate of 1.0 and density of 15 (1.5 foxes/km^2) favors persistence.

of the cycle such that only incubation periods of about 1 month produced 3-4 year cycles.

The final factors that appear to affect persistence are density and contact rate. It is difficult to separate those factors since the number of contacts between animals depends on social behavior which in turn depends on density. We have discovered, however, that only fairly narrow ranges of density and contact support persistence. Fig. 8 illustrates the results of 1 set of persistence experiments with the Ontario Rabies Model. It can be seen that rabies will not persist at either low or high densities or either low or high contact values. In other words, low density and low contact does not permit rabies to spread while high density and high contact cause a 'burn out'. The density values supporting persistence (1-2 foxes/km^2) in those experiments are similar to the densities found in southern Ontario (26).

Some factors do not appear to influence persistence in southern Ontario. As discussed previously there is no evidence for short term changes in the virus and for the existence of reservoirs. Long incubation could influence persistence but there is no way to obtain field evidence to demonstrate that. Presumably long range dispersal will produce a similar effect and such movements have been documented. Finally, there is little evidence to demonstrate that species interactions are able to keep the virus in circulation. In Ontario, the principal vectors are the fox (40% of all reported cases) and the striped skunk (26% of all cases). Cross correlation analysis of times series by species demonstrates that incidence in domestic and companion

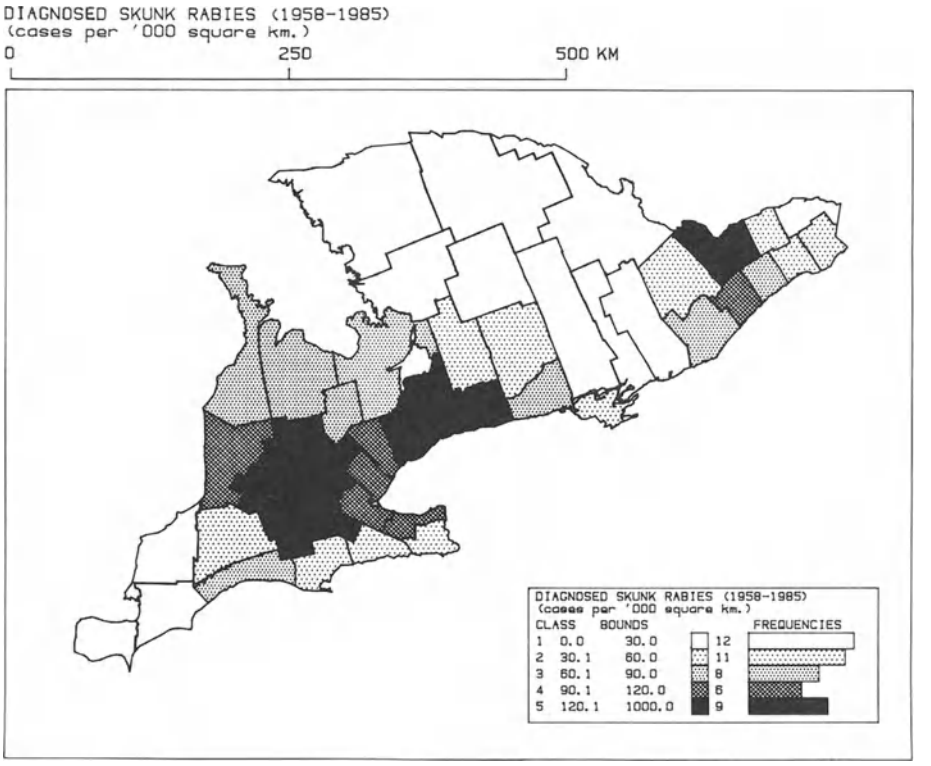

Figure 9. Skunk rabies in southern Ontario by county. Incidence is centered on Ottawa in the east, Toronto to Oshawa in the center and in the Kitchener/Waterloo/Guelph area in southwestern Ontario. Rural areas outside those urban cores have low incidence. Note the absence of skunk and fox rabies (Figure 3) in the flat areas with poor natural drainage in the southern tip of Ontario (near Windsor).

animals lags fox incidence by 1-2 months suggesting that those animals receive virus but do not pass it back into the fox population (36). Similar analysis of fox/skunk series shows little evidence of any relationship between incidence in the 2 species. Skunk rabies is primarily associated with urban areas while fox rabies is more associated with rural areas (Figs. 3,9). Where the 2 species co-exist there is more evidence of fox-to-skunk rather than skunk-to-fox spread. Thus it is unlikely that skunks maintain the virus for subsequent re-entry into the fox population. Unfortunately the analysis of fox/skunk interaction has been at the township level. In 1988, Agriculture Canada will be using a new location code that has a resolution of 100 meters (37). This will allow a more detailed analysis of fox/skunk interaction.

CONCLUSIONS

Many factors have been associated with the maintenance of rabies. No single one of them can explain persistence. In southern Ontario, persistence is a function of the heterogeneity of the habitat within rabies units, the relationship between those units, the reproductive and the movement behavior of the fox population, and the range of behaviors induced by the rabies virus. In southern Ontario and elsewhere, the understanding of persistence will improve when the relationships between the structure of the environment, the host population and the rabies virus are identified. More work must be done on understanding the role of species interactions in maintaining rabies. Finally, more monoclonal studies are needed to identify how the rabies virus adapts over time.

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14

VACCINES AND VACCINATION OF DOMESTIC ANIMALS

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INTRODUCTION

Rabies is one of the oldest recorded diseases of animals, and once signs of the disease appear it is almost invariably fatal. For this reason, the emphasis of early research on rabies was toward the development of vaccines rather than on treatment. Europe was in the midst of a severe rabies epidemic in the 19th century when Pasteur, who had already developed vaccines against fowl cholera and anthrax, began work on vaccines against rabies. The early vaccines were intended for post-exposure treatment of humans, and required multiple inoculations to be successful. Prevention of rabies in man by the vaccination of dogs, the primary reservoir of the 19th century epidemic, was considered impractical because of the need for multiple vaccinations. Instead, laws placing taxes on dogs and requiring the compulsory wearing of muzzles on dogs in large towns were enacted and were successful in some parts of Europe.

EARLY RABIES VACCINES FOR ANIMALS

In 1884, Pasteur and his colleagues first reported on the use of a rabies vaccine in dogs (1). They reported that when virus from a dog was serially passaged intracranially in monkeys, the virus lost its virulence for dogs. Dogs could be inoculated either subcutaneously or under the dura mater (after trephining the skull) without producing disease, and the animals were resistant to subsequent challenge with virulent street virus. However, the vaccine was not completely safe,

and an occasional dog would develop rabies after vaccination. This lack of safety prevented the use of vaccine in man, which was Pasteur's ultimate goal.

Pasteur's next attempt at vaccinating dogs used a serially passaged virus that was further weakened by physical means (3). For use in this vaccination procedure, the spinal cords of rabbits dying after being inoculated with fixed rabies were dried by being suspended for varying lengths of time in bottles containing caustic potash. Dogs could be successfully immunized to subsequent challenge by a series of subcutaneous inoculations starting with nervous tissue preparations that had been made avirulent by drying for the longest period of time. Cord that had been dried for shorter periods and which contained increasing amounts of live virus were injected next. On occasion, dogs would exhibit depression, loss of appetite, nervous symptoms, and paresis of the posterior limbs 8-14 days after the initial vaccination (2). The close association to time of vaccination, and the fact that the animals did not die, suggest that these signs were vaccine-induced sequelae unrelated to the development of rabies.

One of the first vaccines to be widely used in domestic animals was developed by Hogyes in 1888, and his work was summarized by Friedberger and Frohner (2). Rather than using desiccated nervous tissue with little remaining viable virus, Hogyes simply diluted fresh rabbit spinal cords in saline and began the inoculation with the most dilute preparation. The vaccine procedure still required 6 injections which made it impractical for pre-exposure immunization, but it was used extensively in animals that had been exposed to rabies. In more than 15,000 domestic animals of various species receiving post-exposure vaccination, the rate of failure was only 1.5% (4).

Mass vaccination of dogs as a method of rabies control did not occur until 1919. It was then that Umeno and Doi (5) began using a rabbit brain and cord vaccine that was prepared in glycerin and partially inactivated with phenol. A single injection of vaccine appeared to provide adequate protection, but the residual viable virus caused several cases of vaccine-induced rabies. Consequently, production methods for phenolized veterinary vaccines were modified to the method described by Semple (6) to insure complete inactivation.

Table 1. Rabies Vaccine Strains

Modified live virus	Inactivated nervous tissue origin	Inactivated cell culture origin
Flury	CVS	CVS
SAD	Pasteur	SAD
Kelev	51	Pasteur
	91	Flury

CVS = Challenge Virus Standard strain; SAD = Street Alabama Dufferin, and includes ERA and Vnukovo-32.

Other inactivants such as formalin (7) and chloroform (8) were also used to produce killed rabies vaccines. However, the immunizing capabilities of the various types of vaccines available during the early 20th century varied considerably (9,10). In 1940, Habel developed a mouse potency test for inactivated rabies vaccines which allowed these products to be more accurately standardized (11). The improved quality control procedures played a major role in the improvement of rabies vaccine, and these vaccines were effective in reducing canine rabies when used in local control programs (12).

Even though nervous tissue origin (NTO) vaccines were shown to be effective in controlling rabies, they had the disadvantage of causing a significant number of post-vaccinal nervous system reactions. These reactions ranged from mild and transient paralysis to severe paralysis that often resulted in the destruction of the animal. Because of the safety problems with this type of vaccine, there was a continuous effort to develop better vaccines for domestic animals.

MODERN DAY VACCINES

There have been many types of vaccines produced since Pasteur's original fixed virus NTO vaccine. The vaccines that are available today for use in domestic animals can be divided into 3 groups: modified live virus (MLV) vaccines, inactivated NTO vaccines, and inactivated cell culture origin (CCO) vaccines. Some of the virus strains used to produce the different types of vaccines are presented in Table 1. The availability of vaccine in each group varies from country to country.

Modified Live Virus Vaccines

Flury Strain

An important advancement in the vaccination of dogs was the development of a MLV rabies vaccine propagated in chicken embryos. The virus used in this vaccine was isolated from a girl named Flury who died of rabies in 1939 (13). The virus was serially passaged 138 times in 1 day-old chicks before being passaged in 7 day-old embryonated chicken eggs (14). As the virus was passaged, it became less pathogenic for mice and rabbits inoculated intracerebrally, and for dogs inoculated intramuscularly. Vaccine was prepared from the 40-50th chick embryo passages, and was designated low-egg-passage (LEP) (15). Comparative duration-of-immunity studies in dogs and cattle demonstrated that the immunizing capability of the LEP Flury strain was superior to the older type of inactivated NTO vaccine (16-20). Flury LEP vaccine was considered safe and effective in older dogs, but since it would occasionally cause rabies in young pups (21), cats (17,18), and cattle (20,22), the virus was only approved for use in dogs more than 3 months of age. Because the vaccine was only recommended for use in a single species, it became less popular for use in control programs when equally efficacious vaccines that could be administered safely to a variety of animals were developed. In addition, the numerous cases of vaccine-induced rabies in adult dogs vaccinated with the Flury LEP strain (21,23,24) have caused some countries to no longer recommend its use.

The Flury strain of vaccine was further propagated in embryonated chicken eggs in an effort to develop a safer vaccine that could be administered to cats and cattle as well as dogs (25). At the 176-182nd egg passage, the virus lost its ability to kill adult mice but was still pathogenic for suckling mice. This virus was designated high-egg-passage (HEP). When tested at the 205th egg passage, the virus was also non-pathogenic for dogs and rabbits inoculated intracerebrally. Vaccine produced from Flury HEP virus was safe and immunogenic when inoculated intramuscularly in cats and cattle, but it retained its virulence when administered intracerebrally in these species (25-28). Despite the increased safety of the HEP Flury strain over the LEP strain in cats, there have been cases of vaccine-induced rabies

reported (29,30). Immunosuppression caused by feline leukemia may have been a factor in at least 1 of the cases (30).

As cell culture techniques were developed, the LEP and HEP Flury virus strains were adapted to several cell culture systems to reduce anaphylactic reactions caused by the excessive amount of tissue antigen in chick embryo origin vaccine (31-33). Cell culture origin vaccines have the same high level of immunogenicity as the embryo origin vaccines and can protect dogs for up to 3 years (34,35).

Street Alabama Dufferin Strain

Another rabies virus strain that has been used extensively in vaccine production is the Street Alabama Dufferin (SAD) strain. The virus was originally isolated from a rabid dog in Alabama in 1935 and subsequently maintained for several years by serial passage in mice. The virus was adapted to hamster kidney tissue culture by Fenje in 1960 (36).

The first vaccine produced from the SAD strain was identified as ERA after its developers (Evelyn Gaynor, A. Rockitnicki, and M.K. Abelseth). They first passaged Fenje's virus in chicken embryos and then in porcine kidney cells (37). The virus became avirulent for cattle inoculated intracerebrally, but was still virulent for dogs when given by that route. Vaccine produced from the ERA-SAD strain of virus is effective in dogs, cats, cattle, sheep, goats, and horses (38,39). The superior effectiveness of the vaccine in comparative trials with NTO vaccines (40,41) led to its extensive use in South America to control vampire bat-transmitted rabies (42,43).

The SAD virus has been further passaged in canine and bovine cells to produce other vaccines for dogs and cats (38). In addition, the highly attenuated canine cell-passaged SAD virus was further passaged in baby hamster kidney cells to produce a vaccine that has successfully controlled fox rabies in Europe when administered orally (44,45; see also Wandeler, this volume).

The tissue culture-adapted SAD virus developed by Fenje also served as the source of another vaccine. This virus, designated Vnukovo-32, was developed in Russia by passaging Fenje's virus in hamster kidney cells at 32°C (46). The vaccine is recommended for use in dogs, cats, cattle, horses, sheep, and goats (47).

Like the Flury HEP strain vaccine, SAD vaccine has been associated with cases of vaccine-induced rabies in cats (48,49), and some vaccines have been withdrawn from the market because of the concern for safety. In addition to the problem of vaccine-induced rabies in cats, cases of ataxia have been seen in horses recently vaccinated with SAD vaccine (50,51). Although the vaccine virus has not been isolated from these horses, the common history of vaccination strongly implicates the vaccine as a causative factor in the ataxia.

Kelev Strain

The Kelev strain of rabies vaccine has many of the properties of the Flury HEP strain. The virus was originally isolated from a dog in 1950. The virus was passaged 4 times in mice before being passaged in embryonated chicken eggs (52). Like Flury HEP, the Kelev strain lost its ability to kill adult mice but remained virulent for suckling mice. The virus also became avirulent for dogs when administered intracerebrally. The change in virulence for mice was not as abrupt as with the Flury virus, and occurred gradually between the 26th and 70th passages. Vaccine produced from the Kelev strain of virus is recommended for use in dogs and cattle (47).

Killed Virus Vaccines

Suckling Mouse Brain Origin Vaccines

The early rabies vaccines were inactivated preparations of nervous tissue from adult animals, and post-vaccinal nervous system reactions were common because of an encephalitogen associated with myelin. Since the brains of young animals contain less myelin, Fuenzalida and Palacios developed a vaccine consisting of suckling mouse brain material that significantly reduced the incidence of post-vaccinal reactions (53). The vaccine contained a mixture of 3 rabies virus isolates inactivated by ultraviolet light, and it has been shown to be effective in dogs, cats, and cattle in Latin America (41,47,54). Similar vaccines produced exclusively from the challenge virus standard (CVS) strain and inactivated with beta-propiolactone have been used extensively in dogs and cats in North America. Vaccine produced by the latter method has been shown to protect 100% of the vaccinated dogs from rabies at 3 years (55), whereas the ultraviolet light-inactivated vaccine protected 79% of the dogs challenged after the same length of time (56).

Cell Culture Òrigin Vaccine

Rabies virus was first adapted to a non-nervous tissue culture system by Kissling in 1958 (57). Fenje subsequently reported on the preparation of a formalin-inactivated vaccine from SAD virus grown in hamster kidney tissue culture (58). The vaccine was shown to be efficacious in rabbits but was never manufactured for use in dogs and cats. In 1962, Ott and Heyke adapted their mouse origin CVS strain of rabies virus to hamster kidney tissue culture and inactivated the virus with phenol (59). The vaccine protected 100% of the dogs, and 78% of the cats challenged 1 month after vaccination (60). This type of vaccine has not been proven to be as effective in long-term duration-of-immunity studies (34,61). Inactivated CCO vaccines have subsequently been improved, and those that are available today can adequately immunize dogs and cats for 3 years (62-64). Some vaccines are also effective in horses, cattle, and sheep (63). Most of the rabies vaccines marketed today are killed virus (KV) CCO vaccines.

VACCINATION PROCEDURE

There are many vaccines available today that are efficacious when administered properly, and the 2 most important factors are age and route of vaccination. A higher percentage of dogs vaccinated at 11-16 weeks of age with Flury LEP or HEP vaccine survived challenge than did dogs vaccinated from 5-10 weeks of age (65). In another study with Flury LEP vaccine, 6 month-old dogs responded better to vaccination by producing higher antibody titers and surviving challenge than did younger dogs (66). Similar findings have been seen with KV vaccines (67). The poor immune response in the younger dogs is not a result of interference by maternal antibody, since many of the pups were from seronegative dams with no history of rabies vaccination. In 1 study, interference in the vaccination of calves with SAD vaccine was attributed to maternal antibodies (68). However, the antibody titers measured in the younger calves were extremely low and did not correlate with the titers of the dams. Since calves from non-vaccinated dams were not included in the study, the possibility exists that the poor response in the younger animals was the result of an insufficient immmune response rather than interference by maternal antibody.

Regardless of the cause, it is difficult to establish long lasting immunity in young animals. Vaccination challenge tests have indicated that older animals can be immunized effectively for up to 3 years with 1 dose of vaccine but younger animals cannot. The recommendation in the United States is to vaccinate at 3 months of age or older, and again 1 year later regardless of the duration-of-immunity established for the vaccine (69).

For effective use of rabies vaccines, the route of vaccination is as important as the age of vaccination. Modified live virus vaccines are much more effective when given intramuscularly instead of subcutaneously (35,70). For example, 30/30 dogs given Flury HEP vaccine intramuscularly in 1 study (35) survived challenge 3 years later, whereas only 17/29 dogs vaccinated subcutaneously survived.

The superiority of the intramuscular route is not as evident with inactivated vaccines. However, when NTO vaccine was administered by both routes to foxes, 100% of the animals vaccinated intramuscularly survived, as compared to 65% of those vaccinated subcutaneously (71). In another study in which a KV NTO vaccine was administered to dogs, 97% of the dogs vaccinated by the intramuscular route were protected compared to 78% vaccinated subcutaneously (66). Despite these findings, some KV vaccines can be administered subcutaneously without compromising their immunogenicity, but since there are few such vaccines, the label recommendations should be reviewed thoroughly before any vaccine is administered by that route.

When the intramuscular route is recommended, the site of injection should be confined to the muscles of the thigh. Vaccination into the lumbar musculature has been suggested as an enhancing factor in some cases of vaccine-induced rabies in dogs (21), and vaccination into the cervical muscles in horses has been incriminated in cases of suspected vaccine-induced ataxia (51).

CONCLUSIONS

There are many types of rabies vaccines produced from a variety of viral strains, and their availabilities vary from country to country. Despite the antigenic differences among rabies strains demonstrated by monoclonal antibodies (72; see also Smith and Baer, this volume),

immunization by giving vaccine of proven potency by the correct route will protect against infection by different rabies strains (73). They may not, however, provide adequate protection against the rabies-related viruses such as Mokola (see King and Crick, this volume). Duration of immunity and safety are more important in selecting a vaccine than is the vaccine strain used in production. Vaccines that will provide 3 years of immunity are preferred because their use constitutes the most effective method of increasing the proportion of immunized animals in rabies control programs. Neuro-allergenic reactions can be prevented by the use of CCO vaccines, and vaccine-induced rabies can be prevented by the use of inactivated vaccines. Regardless of the vaccine used, it must be administered properly to provide the desired protection.

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DEVELOPMENT OF A VACCINIA-RABIES GLYCOPROTEIN RECOMBINANT VIRUS VACCINE

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INTRODUCTION

Despite its antiquity as a major zoonosis, and the firm recognition of its salient biological features by the late 19th century, rabies commands considerable public health and scientific attention to the present day. Unquestionably, neonatal rotaviral diarrhea, malaria, schistosomiasis, filariasis, foot and mouth disease, brucellosis, leptospirosis, and a multitude of other infectious diseases exact greater global mortality and economic loss (1), but few wield a similar specter of hysteria or prognostic dilemma as rabies, especially once the neurological syndrome manifests.

Except for Antarctica and Australia, animal rabies remains entrenched on all continents, albeit at an obvious dichotomy between the developed and developing countries. For example, 87% of the total 7,946 cases reported in the U.S.A. and Canada during 1985 were diagnosed in wildlife, whereas more than 90% of the 10,756 cases reported in Mexico during the same time period were attributed to canine rabies (2). Practical long term global solutions to both urban and sylvatic rabies control will be achieved by modern adjuncts to traditional rabies interventional measures, one of the most attractive methods consisting of oral immunization.

Extensive field trials in large endemic fox rabies areas of Western Europe with an attenuated rabies virus vaccine highly suggest that oral vaccination campaigns are both feasible and cost effective, and may actually invoke a previously unthinkable concept: local eradication of terrestrial rabies (3,4; Wandeler, this volume). Success

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of any wildlife or feral dog rabies control program that exploits such a strategy, however, is ultimately dependent upon a major keystone: the efficacy, safety, and cost of a distribution system for vaccine-laden bait intended for target species consumption under the vagaries of field conditions. As such, the "ideal" rabies vaccine (i.e., non-hazardous, enduring potency, inexpensive, thermostable up to 40°C, effective orally for diverse mammalian fauna, etc.) does not exist among available commercial rabies vaccines produced by conventional methodologies. Alternatively, vaccinia virus (VV), a member of the Poxviridae, has been intensively studied at the molecular level, partially due to its intrinsic involvement in the successful international smallpox eradication campaign. Although its exact pedigree remains uncertain, VV is closely related to other orthopoxviruses (i.e. cowpox, variola, ectromelia, etc.), yet is sufficiently distinct on the basis of ultrastructural, serological and DNA sequence criteria to be considered a unique virological species. Recent molecular and biotechnological advances have permitted the development of VV as an efficient cloning and expression vector system (5,6); recombinant VV bearing foreign protein coding sequences have been widely used experimentally (7,8).

Vaccinia virus is particularly attractive for foreign gene expression since it has a large DNA genome capable of readily accepting additional genes (9-11), circumventing packaging constraints imposed upon smaller viruses, and is relatively innocuous to the vaccinated host (12-15). Live VV recombinants expressing foreign genes for surface antigens of a variety of pathogens (e.g. herpes, hepatitis, VSV, etc.) demonstrate protective immunity in laboratory animals upon challenge with the corresponding agent, without observable secondary clinical signs attributed to recombinant virus (11,12,16-22). Utilizing these basic data on the molecular biology of VV, and determination of the specific nucleotide coding sequence of the rabies viral glycoprotein (G), permitted the development of a vaccinia-rabies glycoprotein (V-RG) recombinant virus (23-28) specifically containing the G gene of rabies virus correctly expressing the rabies viral G protein, capable of inducing rabies-specific virus-neutralizing antibodies (VNA), and conferring protection against rabies in the inoculated host.

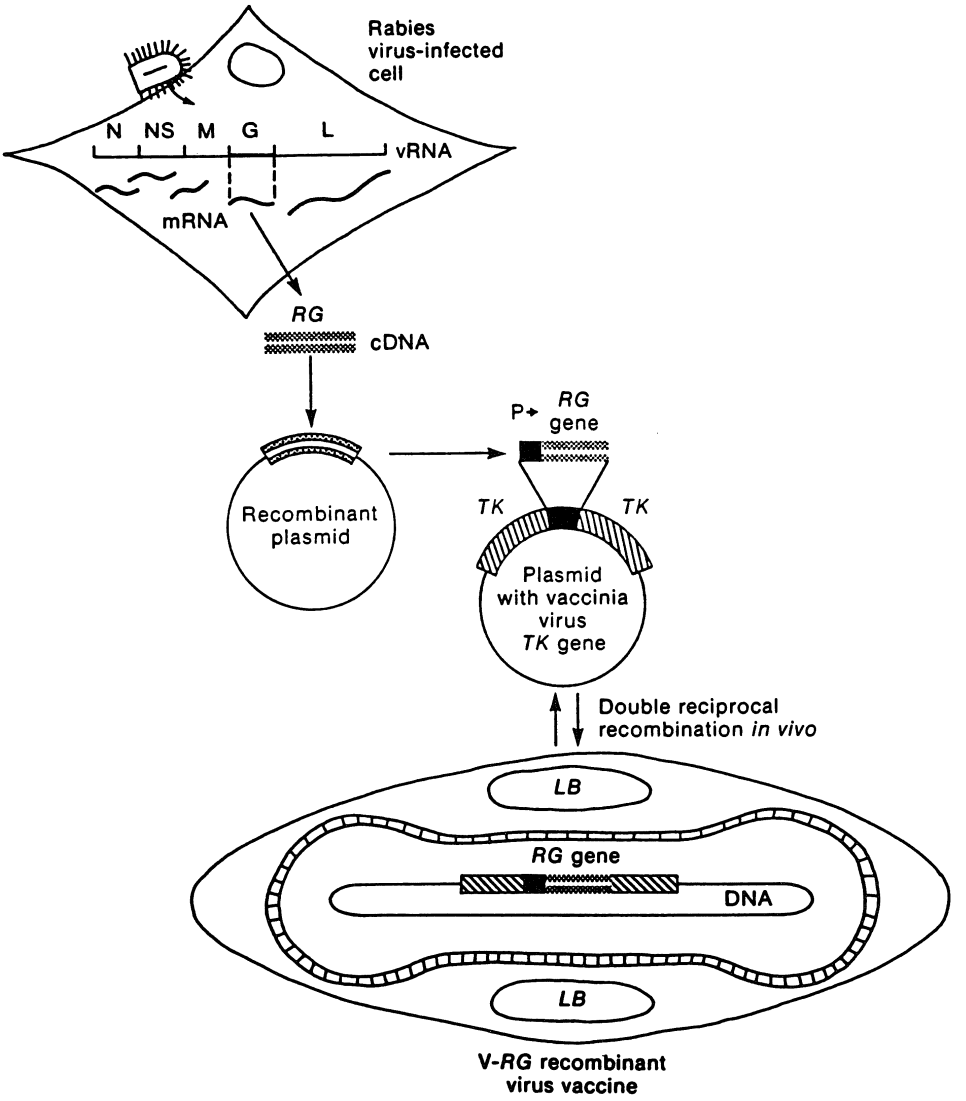

Figure 1. Schematic representation of the construction of the V-RG recombinant virus. Abbreviations: RG, rabies viral G protein; P, vaccinia E 7.5K promoter; TK, vaccinia thymidine kinase gene; LB, vaccinia lateral body. At bottom, the recombinant genome is depicted within the core of the vaccinia virus vector. See text for further details. Figure kindly supplied by Dr. W.H. Wunner.

This review summarizes the efficacy and safety results obtained to date with V-RG vaccine. The data reported herein were obtained from captivity studies conducted on a diverse array of animal species by the Wistar Institute of Anatomy and Biology (Philadelphia, PA, U.S.A.), Transgène (Strasbourg, France), Rhône Mérieux (Lyon, France), the Centre National d'Etudes sur la Rage et la Pathologie des Animaux Sauvages (Malzéville, France), the Faculté de Médecine Vétérinaire de l'Université de Liège (Belgium), and the Animal Diseases Research Institute (Nepean, Ontario, Canada).

V-RG DEVELOPMENT

The rabies virion consists of 5 virus-encoded proteins (N, NS(M_1), M(M_2), G and L), of which only 1, G, traverses the lipid bilayer envelope and is capable of eliciting rabies VNA and conferring protection against rabies (see Tordo and Poch, this volume). Given its previous history as an efficient recombinant and cloning system, we explored the use of a VV vector to express the fixed rabies virus ERA coding sequence (24).

Expression of an RNA coding sequence in VV involves 3 basic steps: conversion to a cDNA, plasmid construction, and transfection. The intended gene (as cDNA) is first inserted downstream of a VV promoter into a non-essential segment of VV DNA in a plasmid vector. Double reciprocal recombination *in vivo* between this plasmid and the VV genome secondarily permits integration of the DNA insert into the viral genome (Fig. 1).

Restructuring of the Rabies G Coding Sequence

In the cloned rabies G-coding sequence of Anilionis *et al.* (23), the ATG corresponding to the initiation codon of the message lies adjacent to a poly(dG) sequence introduced by the cDNA cloning procedure. Since a second poly(dG) track is positioned at the end of the cDNA for the same reason, this was likely to generate instability of the rabies gene in VV. The deletion of the first poly(dG) sequence of the cDNA was accomplished using a double-stranded oligonucleotide to link an outside *Bgl* II site to a unique *Mst* II site overlapping codons 2,3,4 (Fig. 2a).

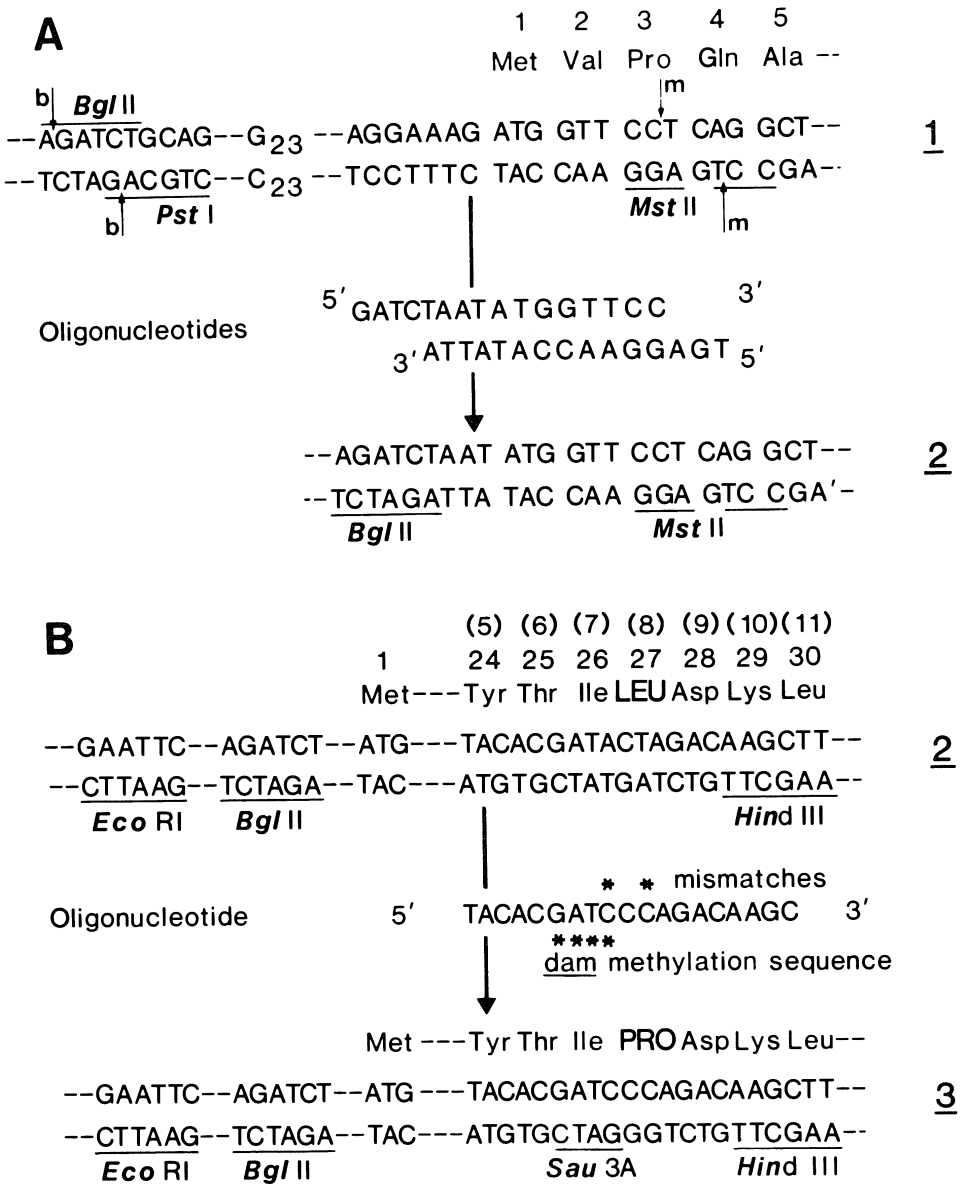

Figure 2. Restructuring of the rabies G-cDNA (ref. 24). **A.** Elimination of the 5' d(G) sequence. **B.** Correction of leucine 8 to proline. Numbering of the codons/amino acids starts with the signal peptide (residues 1-19) necessary for glycosylation of the G protein. Sequence of the G protein itself (numbers in brackets) starts at position 20.

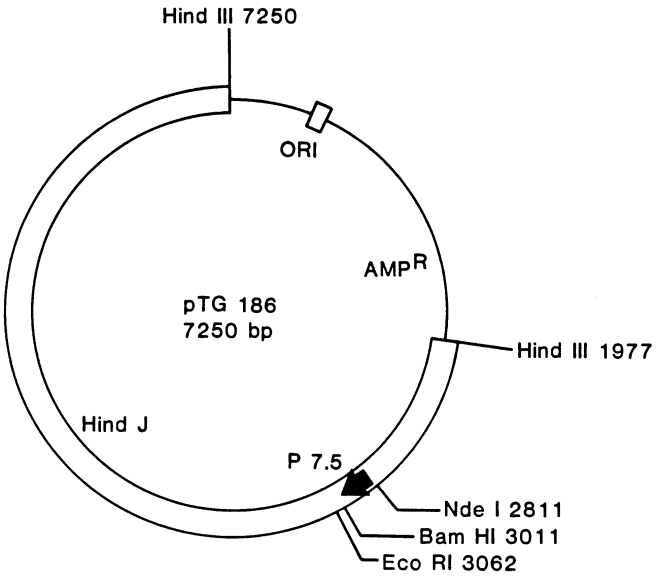

Figure 3. Structure of plasmid pTG186. Amp^R: ampicillin resistance gene; ORI: origin of replication of plasmid pBR322; P7.5: 7.5K gene promoter; Hind J: *Hin* dJ restriction fragment of VV genomic DNA which contains the TK gene. From ref. 24, with permission.

In the cloned ERA virus G-coding sequence, the 8th codon of the mature G sequence is for leucine, whereas in the actual G protein of both the ERA and CVS strains of rabies virus this amino acid is a proline. The presence of a leucine instead of a proline led to aberrant processing of the G protein (27). Therefore, the 5' extremity of the G-cDNA was subcloned into bacteriophage M13 and the leucine codon (CTA) corrected to a proline codon (CCA) (Fig. 2b) using oligonucleotide site-directed mutagenesis. The corrected segment was subsequently repositioned in the rabies G-cDNA.

Plasmid Construction

In pTG186 (also named pTG1H-TK-P7.5K in ref. 24), a unique *Bam* HI site lies immediately after the VV 7.5K promoter. The structure of this plasmid is given in Fig. 3. The thymidine kinase (TK) gene of VV was chosen as a non-essential segment of VV DNA, disruption of which results in a TK$^-$ phenotype. In medium containing HAT (hypoxanthine-

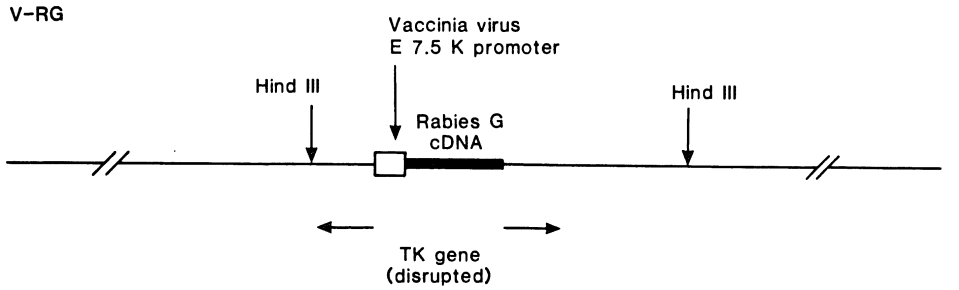

Figure 4. Diagrammatic structure of the V-RG genome. Shown is the TK region of the vaccinia genome with the inserted rabies G-cDNA from *Hin* dIII-digested plasmid pTG187-PRO (see Fig. 3).

aminopterin-thymidine), growth of TK^+ phenotypes are inhibited whereas TK^- phenotypes are not. Culture *in vitro* in this medium, therefore, provides a simple means for selection of TK^- recombinants.

In plasmid pTG155-PRO (24), the restructured rabies G-cDNA is flanked by *Bgl* II sites; this segment was introduced into the *Bam* HI site of pTG186, generating plasmid pTG187-PRO (pVVTGgRAB in ref. 24).

Cloning into VV

The strategy devised by Panicali *et al.* (5) to generate VV recombinants relies on the *in vivo* exchange between a plasmid bearing an insert within a VV gene and the wild-type viral genome.

Since VV DNA is non-infectious, it is necessary to infect cells with live VV and simultaneously introduce cloned DNA by calcium-mediated transfection. This technique was used to transfer the rabies G expression block to the VV (Copenhagen strain) genome, creating V-RG (VVTGgRAB26D3 in ref. 24) (Fig. 4).

Infection of cell cultures with the V-RG recombinant elicited the production of a correctly processed rabies G protein which reacted strongly with rabies-neutralizing monoclonal antibodies. Indeed, the profile of reactivity of the recombinant rabies G with a panel of monoclonal antibodies was largely identical to that obtained with native ERA virus, attesting to the authenticity of the recombinant G protein (24).

CONTAINMENT TRIALS OF V-RG RECOMBINANT VIRUS

Laboratory Animal Species

Mouse

The preliminary trials concerning the safety and efficacy of V-RG virus were initially conducted in laboratory animals by parenteral routes of vaccine administration. The V-RG recombinant virus was tested in both inbred (A/J) and outbred (ICR) strains of laboratory mice using both live and beta-propiolactone (BPL)-inactivated vaccine preparations (27). Immunization was performed by footpad inoculation and intradermal (i.d.) tail scarification with either wild-type VV or V-RG virus (10^9 PFU/ml). Inoculation of mice with V-RG virus resulted in the relatively rapid induction of rabies VNA, reaching titers of at least 30,000 units within 2 weeks (Table 1), but lesser titers against wild-type VV itself. A strong secondary cytotoxic T lymphocyte response specific for rabies G was also prominent (27). All mice

Table 1. Induction of VNA in Mice and Protection from Rabies by V-RG

Inoculation Route	Vaccine[a]	VNA (GMT)[b]			Protection[c]
		Rabies		Vaccinia	
		Day 0	Day 14	Day 14	
i.d.	V-RGpro8	<10	>30,000	250	12/12
	V-RGleu8	<10	<10	-	0/12
	Vaccinia	<10	<10	250	0/12
Footpad	V-RGpro8	<10	>30,000	1,250	12/12
	V-RGleu8	<10	<10	-	0/12
	Vaccinia	<10	<10	1,250	0/12

[a] Vaccine was inoculated on day 0 using 2×10^8 PFU (i.d.) or 5×10^7 PFU (footpad).

[b] Neutralizing VNA geometric mean titers (GMT) as noted throughout are expressed as the highest serum dilution that reduced the number of virus-infected cells by 50% in the rapid fluorescent focus inhibition test (RFFIT) (rabies) or in plaque reduction (vaccinia) assays.

[c] A challenge dose of 2,400 $MICLD_{50}$ of MD5951 rabies virus was given i.c. on day 14; number of survivors/ number challenged.

(Adapted from ref. 27).

Table 2. Dose Response of Mice Immunized with V-RG Vaccine[a]

Dose (log_{10}PFU)	Rabies VNA (GMT)		Protection
	Day 7	Day 14	
7.7	2,400	1,600	10/10
6.7	2,400	>30,000	10/10
5.7	2,400	>30,000	10/10
4.7	800	4,000	8/10
3.7	20	3,000	4/10
2.7	<10	<10	1/10
1.7	<10	<10	0/10
Controls	<10	<10	0/10

[a] All mice were seronegative for rabies VNA on day 0 when they were inoculated with 0.2 ml serial dilutions of V-RG intra-footpad. Mice were inoculated on day 15 i.c. with 2,400 $MICLD_{50}$ of street rabies strain MD5951.

(Adapted from ref. 27).

receiving V-RG resisted challenge on day 14 with street rabies virus by intracerebral (i.c.) inoculation of 2,400 $MICLD_{50}$, and were observed for a minimum of 3 months thereafter. Mice similarly immunized with wild-type VV alone were unprotected. Unlike the proline recombinant virus (V-RGpro8), the V-RG construct expressing leucine at position 8 of the rabies G sequence (V-RGleu8) was ineffective in either the induction of high rabies-specific VNA or protection against i.c. rabies challenge. The potential of V-RGleu8 virus against less severe but more natural routes of rabies exposure (i.e. peripheral challenge) and the relative protective role of cell-mediated effector mechanisms in lieu of high VNA levels with this particular construct, was not evaluated and subsequently eliminated from further testing.

The minimum effective dose of V-RG virus capable of protecting at least 50% of recipient mice inoculated in the footpad and challenged i.c. after 15 days was approximately 10^4 PFU (27) (Table 2). In 2 additional trials, when BPL-inactivated virus was used, 12 mice were inoculated with 2 intraperitoneal (i.p.) inoculations (0.5 ml) 7 days apart, and subsequently challenged with 240 $MICLD_{50}$ of lethal rabies virus on Day 14. All inactivated V-RG preparations induced high levels

Table 3. Induction of VNA in Mice and Protection from Rabies by Inactivated V-RG

Vaccine[a]	Titer before inactivation (log_{10}PFU/ml)	Protein conc.[b] (ug/mouse)	Rabies VNA (GMT)		Protection[c]
			Day 7	Day 14	
V-RG virus-infected cell extract	7.5	140	80	8,000	12/12
V-RG sucrose gradient purified virus	8.6	9	270	4,000	12/12
Vaccinia virus-infected cell extract	8.6	900	<10	<10	0/12
Unvaccinated Controls	-	-	-	<10	0/12

[a] Vaccines were prepared from infected BHK-21 cells, inactivated with BPL, and inoculated i.p. on days 0 and 7.

[b] Total protein in 2 i.p. inoculations given on days 0 and 7.

[c] Challenged i.c. with 240 $MICLD_{50}$ of MD5951 rabies virus on day 14.

(Adapted from ref. 27).

Table 4. Protective Immunity in Mice from V-RG Vaccine: Effect of Challenge Virus and Duration Post-vaccination[a]

Challenge virus	V-RG vaccine concentration (log_{10}PFU)	Protection
Duvenhage	5.7	8/10
	5.0	8/10
	4.3	6/10
Controls	-	0/10
CVS-24	5.7	9/10
	5.0	9/10
	4.3	9/10
Controls	-	0/10

[a] Four to 6 week-old female ICR mice were inoculated i.m. with 0.1 ml of V-RG on days 0 and 7 and challenged either on day 14 with 0.03 ml of Duvenhage virus i.c. (10^5 $MICLD_{50}$) or at 16 weeks with 0.1 ml of CVS virus i.m. ($10^{6.2}$ $MICLD_{50}$).

Table 5. Induction of VNA in Rabbits and Protection from Rabies by V-RG

Inoculation route	Vaccine[a]	VNA (GMT)					Protection[b]
		Rabies				Vaccinia	
		Day 0	Day 5	Day 11	Day 14	Day 14	
i.d.	V-RGpro8	<10	800	10,000	>30,000	250	3/4
	V-RGleu8	<10	-[c]	-	>10	-	-
	None	<10	-	-	>10	-	0/5

[a] Vaccine was inoculated on day 0 using 2 x 10^8 PFU.

[b] Challenged with 24,000 $MICLD_{50}$ of MD5951 rabies virus on day 14.

[c] Not done. (Adapted from ref. 27).

of rabies VNA and protected mice against severe rabies challenge (Table 3), implying that the rabies viral glycoprotein is actively associated with the V-RG recombinant virion.

On the basis of circulating VNA titers alone, the potential duration of immunity of V-RG virus in the mouse model exceeds 24 weeks (Rupprecht, unpublished data), and at least 90% of mice challenged peripherally with CVS-24 at 16 weeks were subsequently protected (Table 4). Immunity produced by the V-RG vaccine is not limited to those terrestrial street viruses complementary to ERA G antigenic structure. All mice immunized via footpad with 5 x 10^7 PFU of V-RG vaccine resisted day 14 challenge with $10^{3.4}$ $MICLD_{50}$ of American bat rabies and African Duvenhage lyssavirus strains; 80% of mice immunized i.m. with $10^{5.0}$ PFU of V-RG survived i.c. European Duvenhage (Duvenhage 6, Polish bat origin) virus challenge (Table 4). In contrast, no V-RG immunized mice (0/12) were protected against i.c. challenge with rabies-related Mokola lyssavirus.

Syrian Hamster

Given its pre-exposure efficacy, V-RG virus was assessed in a post-exposure rabies virus scenario. Groups of 12 Syrian hamsters were inoculated i.m. with 0.1 ml of either live (10^7 PFU/ml) or of BPL-inactivated, sucrose gradient-purified, V-RG vaccine (antigenic value 5.7 IU/ml) (28) at 24 hr prior to, or at 1 hr and 3 days following i.m. challenge with $10^{1.2}$ hamster LD_{50} of rabies street virus strain MD5951.

This was compared to hamsters receiving an inactivated PM-Vero cell rabies vaccine (antigenic value 3.3 IU/ml) or saline controls similarly inoculated. All controls and 67-83% of PM-immunized hamsters succumbed. In contrast, 42-50% of hamsters immunized with inactivated V-RG, and 50-83% inoculated with live V-RG vaccine survived challenge, attesting to its potential utility in veterinary pre- or human post-exposure protocols, pending additional study with and without rabies immunoglobulin.

Rabbit

New Zealand White rabbits were inoculated i.d. with 2×10^8 PFU of live V-RG virus distributed into 3 separate shaven sites on the dorsum. Rabies VNA titers at 5, 11, and 14 days after inoculation were 800, 10,000, and >30,000, respectively; sera obtained from V-RG immunized rabbits effectively neutralized between $10^{5.3}$-$10^{6.6}$ $TCID_{50}$ of several antigenically distinct bat and fox street rabies and African Duvenhage viruses, but not substantially the rabies-related Lagos bat and Mokola lyssaviruses. As with mice, wild-type V-RGleu8 virus was ineffective in elicitation of rabies VNA. Also, VV VNA titers after 14 days were substantially lower (27). The immunized rabbits were challenged with street rabies virus by i.c. inoculation with 24,000 $MICLD_{50}$, and were observed for a minimum period of 3 months. Three of 4 rabbits vaccinated with V-RG virus resisted challenge. All 5 unvaccinated control rabbits died from rabies after 12-15 days (Table 5). The single vaccinated rabbit succumbing to rabies survived until 21 days post-challenge.

A secondary response to V-RG recombinant virus inoculation was tested to assess whether primary (previous) vaccination interfered with the immune response to a second inoculation of the same VV recombinant vaccine. Three rabbits immunized i.d. with $10^{7.6}$ PFU of V-RG virus and showing a VNA titer >30,000 at 15 days post-vaccination, were subsequently inoculated i.d. 6 months later with the same dose of V-RG virus. Twenty-one days after the booster, the rabbits were challenged i.c. with 2.4×10^4 $MICLD_{50}$ of street rabies virus. Following the booster inoculation, the levels of VNA in all 3 animals increased dramatically starting on day 3 (titers 24,000) and reached titers of 70,000 or higher by day 15 (Table 6). All animals resisted challenge

Table 6. Booster Response[a] of Rabbits to V-RG Virus

	Rabies VNA titers				
Animal #	Day 180	Day 183	Day 185	Day 195	Protection
1	8,000	24,000	70,000	>70,000	+
2	8,000	24,000	70,000	>70,000	+
3	12,000	24,000	>70,000	>70,000	+

[a] Primary inoculation occurred on day 0 using $10^{7.6}$ PFU of virus i.d., with a booster (same dose and route) at 6 months: i.c. challenge with 24,000 $MICLD_{50}$ of rabies strain MD5951 occurred within 21 days.

(Adapted from ref. 28).

with street rabies virus (28). These results indicate that primary immunity induced by V-RG recombinant virus does not interfere with the systemic response to the same immunogen given 6 months later.

Somewhat surprisingly, rabies VNA titers following vaccination with 0.2 ml of V-RG recombinant virus ($10^{7.6}$ PFU) by the subcutaneous (s.c.), intramuscular (i.m.), or oral routes (animals were induced to swallow the vaccine) were higher than those following i.d. inoculation. Protection after rabies street virus challenge was total (Table 7) (28), as was post-vaccinal safety (except for very limited local skin reactions after inoculation via the i.d. route).

Target Wild Animal Species

Raccoon

Over the past 40 years, the raccoon (*Procyon lotor*), an especially ubiquitous and abundant mammalian carnivore, has become a prominent rabies reservoir in the southeastern and mid-Atlantic regions of the U.S.A. (2), prompting laboratory trials with V-RG aimed at effective oral immunization, since conventional attenuated rabies viruses had proved ineffective. Adult raccoons maintained in captivity received 1 ml (10^8 PFU) of V-RG virus contained in a 3 cm^3 polyurethane sponge coated with a beef tallow/paraffin wax mixture (29), by ingestion. Any animal not eating a vaccinia-laden bait within 48 hrs was given 1 ml of either 10^8 or 10^6 PFU of V-RG recombinant virus by oral infusion (30). Controls received placebo baits or virus-free cell culture media. Of 20 raccoons given V-RG recombinant virus in sponge

Table 7. Immune Response of Rabbits to V-RG virus Administered via Different Routes[a]

Inoculation route	Rabies VNA titers (GMT)						Protection
	Day 0	Day 3	Day 5	Day 7	Day 15	Day 21	
i.d.	<3	6	3,500	7,200	8,500	24,000	+
i.m.	<3	45	7,200	48,000	48,000	135,000	+
s.c.	<3	45	7,200	70,000	70,000	100,000	+
Oral	<3	<3	2,400	24,000	70,000	115,000	+
None	<3	-	-	-	-	<3	+

[a] Vaccine was inoculated on day 0 using $10^{7.8}$ PFU of V-RG to pairs of rabbits which were challenged i.c. on day 21 with 24,000 $MICLD_{50}$ of rabies virus strain MD5951.

(Adapted from ref. 28).

bait, 18 ingested the bait within 48 hrs of presentation. One of 2 animals that did not eat the bait was given an oral infusion of 10^6 PFU/ml and the other received 10^8 PFU/ml V-RG virus. All animals developed rabies-VNA following oral infusion or ingestion of bait containing V-RG recombinant virus (Table 8). Seventeen of 20 animals immunized with V-RG virus and challenged i.m. 28 or 205 days after immunization with $10^{5.5}$ $MICLD_{50}$ survived, and 16 showed a prominent anamnestic VNA response 30 days after challenge. The animal that received 10^6 PFU of V-RG virus by oral infusion and 2 of 10 animals that ate the bait (but were challenged 6 months after immunization) succumbed. Seroconversion rates of raccoons following V-RG virus inoculation by a variety of routes was compared and no differences were apparent in the ability of animals to resist lethal challenge with rabies virus. Vaccine efficacy was apparently not enhanced by buccal scarification or administration of booster doses (31) (Table 8). Thus, while the initial use of V-RG by the oral route was conducted in laboratory rodents (31) and rabbits (28), both this vaccine and route held great promise for control of rabies in relevant carnivore models.

Several experiments were conducted to investigate the potential roles of horizontal or vertical transmission of V-RG virus in raccoons. In cage trials with pairs of adult raccoons, 2 of 5 non-immunized contact animals developed low rabies VNA levels and survived rabies

Table 8. Immunization and Protection from Rabies in Raccoons by V-RG

Route	Dose (PFU/ml)	No. of animals	Rabies VNA titers[a]	Day of challenge[b]	Protection
i.d.	$10^{7.0}$	3	600-1,215	28	3/ 3
i.m.	$10^{7.8}$	3	405-1,215	63	2/ 3
Oral	$10^{6.0}$	1	90	28	0/ 1
Oral	$10^{8.0}$	6	45-3,645	28	6/ 6
Oral (booster dose, day 42)	$10^{7.8}$	1	450	63	1/ 1
Oral (buccal scarification; booster, day 42)	$10^{7.8}$	2	450	63	1/ 2
Sponge baits	$10^{8.0}$	8	45-3,645	205	8/ 8
		10	45-1,215	205	8/10
Oral (Inactivated)	-[c]	6	<15	28	0/ 6
Oral (controls)	0	17	<15	28-205	1/17

[a] Range of rabies VNA titers determined 16-28 days post-immunization.

[b] Inoculated i.m. with $10^{5.5}$ $MICLD_{50}$ of rabies street virus MD5951 on the days indicated post-vaccination.

[c] Titer $10^{7.2}$ PFU/ml before inactivation.

(Adapted from refs. 30,31).

virus challenge as did the other cagemates actually immunized with V-RG virus orally. Only the male-female pair combinations displayed any evidence of contact transfer of virus, but the limited number of animals in this study did not allow a definitive interpretation of results (31). With regard to pregnancy, 2 adult female raccoons immunized with 1 ml (10^7 PFU) of V-RG recombinant virus within 30 days of parturition gave birth to healthy litters of 3-4 kits each. All littermates had levels of rabies VNA at birth comparable to that of the adult females (titers approximately 135), suggesting the occurrence of either passive transfer of maternal antibody or active V-RG transmission *in utero*. No virus could be isolated from the young animals (31).

In a separate observation, 3 suckling raccoons approximately 3-4 weeks old were replaced with the dam immediately after the adult female received 1 ml (10^7 PFU) of V-RG recombinant virus by mouth. All animals remained healthy, seroconverted within 28 days, and survived peripheral rabies virus challenge. The actual transmission route in this study (e.g., via lactation, grooming, etc.) was not determined.

In a related V-RG transmission experiment, 4 adult free-ranging pregnant raccoons (diagnosed as having 2-3 kits each *in utero* by manual palpation and ultrasonography) were live-trapped in a terrestrial rabies-free area of southeastern Pennsylvania, U.S.A. One female gave birth to 3 healthy kits and was given 1.0 ml ($10^{7.8}$) of V-RG for consumption in a sponge bait 2 days post-partum. The remaining 3 raccoon females were sedated, bled for rabies VNA, and given the same V-RG inoculum directly, *per os*. These each gave birth to 3 healthy kits 2, 8, and 20 days post-immunization. All females and their offspring remained healthy over the next 3 months. At that time, all animals were sedated, bled, and inoculated i.m. with a previously determined sub-immunogenic dose (1.0 µg) of BPL-inactivated, purified ERA rabies vaccine. Three days later, serum for VNA and peripheral blood lymphocytes were obtained by venipuncture, the latter for determination of cell proliferative indices (CPI) by scintillation spectroscopy with [^{3}H]-thymidine.

Following V-RG immunization, all adult females had systemic VNA (GMT, 0.5 IU/ml) at the time of peripheral inactivated rabies vaccine booster, as did the kits. The 3 days pre-, and 2 days, 8 days, and 20 days post-partum groups had GMT of VNA at 0.7, 10.9, 5.6 and 18.8 IU/ml, respectively. However, only the adult raccoons were sufficiently primed to demonstrate an anamnestic response from the vaccine booster (>4-fold rise in VNA, or a significant increase of CPI over the baseline incorporation of [^{3}H]-thymidine in the absence of antigen) (C. Rupprecht and E. Celis, unpublished data).

If one assumes the reproductive physiology of raccoons is similar to that of better-studied carnivores, it is reasonable to expect that passive transfer of immunoglobulin to offspring does not occur to a significant extent *in utero*. Rather, VNA may be transferred via colostrum during a relatively narrow temporal window (24-48 hrs post-

partum), and newborn raccoons, while partially immunocompetent, possess a comparatively immature immune system for the first 1-3 months of life. If so, we may conclude that kits greater than 1 month of age may acquire active immunization by V-RG/bait contact or secondary grooming contact from a recently immunized dam. However, there was no evidence of active *in utero* or lactogenic transmission of V-RG in the pregnant or recently parturient raccoon females to their offspring; such kits most likely demonstrate passive immunity only, which may be protective against subsequent rabies infection. Moreover, no abortifacient or detrimental characteristics of V-RG were grossly evident either upon pregnant and lactating raccoons or their offspring during an otherwise critical physiological period.

During sequential pathogenicity studies, V-RG recombinant virus was recovered only from buccal mucosa, tonsils, and submandibular/parotid lymph nodes of orally-immunized raccoons during the first 48 hr of vaccination. No viremia was detected during 14 days of observation post-inoculation, no gross or histopathologic lesions suggestive of bacterial or viral infection were found in any of the sampled tissues, and no evidence for persistent infection was documented (31).

Red Fox

The rabies cycle in Western Europe is primarily maintained by the red fox (*Vulpes vulpes*) (see Blancou, this volume). Whereas prophylactic control measures such as culling of foxes have proven only moderately successful in slowing advancing rabies fronts, vaccination in the wild represents a more effective countermeasure (3,4). To this end, European foxes (*Vulpes vulpes*) captured and raised in captivity were inoculated with live V-RG by several routes: i.d., s.c., oral (32). No adverse generalized systemic or local reactions were observed in any animal regardless of inoculation route, and the mild cutaneous inflammation at the site of i.d. inoculation regressed spontaneously within 7 days. Additional foxes received live V-RG virus by direct administration in the mouth or in baits (consisting of 1.8 ml of V-RG virus sealed into plastic blister-packages - a gift from Dr. A. Wandeler) inserted into a chicken head, with 1 blister-package/head and 1 chicken head/fox. None of the animals showed any impairment of digestive function after ingestion of the vaccine.

All animals vaccinated with 10^8 PFU of V-RG survived an i.m. challenge of $10^{4.2}$ fox LD_{50} units at day 28 post-immunization. The VNA titer at day 28 was equivalent for all routes of vaccine administration (~320 units), except for 1 animal inoculated s.c. which did not develop detectable VNA.

The potential horizontal transmission of V-RG virus was also investigated. Four foxes were vaccinated by the oral route with 10^8 PFU of V-RG. Each was housed in the same pen as an untreated animal of the opposite sex. Only 1 of the 4 non-immunized contact animals developed a significant VNA titer. It was noticed that this particular pair of animals exhibited especially aggressive behavior and that the contact subject (female) was bitten by the vaccinated fox (male) immediately after the administration of the vaccine. This seropositive female was subsequently protected against i.m. rabies virus challenge.

A clear dose-response was observed in foxes receiving less than 10^8 PFU of V-RG by the oral route. For example, 1/4, 6/8, 4/4 and 10/10 animals survived rabies challenge after oral administration of 10^4, 10^6, 10^7 and 10^8 PFU of V-RG, respectively. These results indicate that 10^5 PFU of V-RG are necessary to vaccinate 50% of the foxes by the oral route. This dose is only 10 times greater than the minimum dose capable of protecting 50% of mice by footpad inoculation (see preceding section).

To evaluate the duration of immunity, groups of foxes were challenged at various times after vaccination (4 animals in each group, 6, 12 and 13 months post-vaccination). Although the VNA titer rapidly decreased between 1 and 3 months after vaccination, all animals survived i.m. rabies challenge, except 2 animals in the 6 month group, in which some problems were experienced with the uptake of the vaccine (J. Blancou, personal communication). This indicates that immunity conferred by the V-RG vaccine is of relatively long duration, as animals were still resistant to rabies challenge 18 months after vaccination.

Fox cubs

Thirteen wild foxes aged between 6 and 12 wk were captured from a rabies-free area and vaccinated with $10^{7.2}$ PFU of V-RG by direct application into the mouth. On day 28, all animals but 1 had titers

of rabies VNA ranging between 0.9 and 21 IU/ml (GMT: 5.7 IU). Groups of 5, 4, and 4 foxes were challenged i.m. on day 33, 180 and 360 respectively with $10^{3.2}$ $MICLD_{50}$ of a fox-adapted street rabies virus. All animals challenged at day 33 and 180 survived. In the group of foxes inoculated with rabies virus on day 360, 2 were protected, 1 died accidentally, and 1 animal which had never presented any detectable rabies VNA died from rabies (P.-P. Pastoret, personal communication). None of the fox cubs showed any clinical signs after V-RG vaccination, attesting to the innocuity of the recombinant virus, and demonstrating V-RG efficacy in the immature animal.

Striped Skunk

The striped skunk (*Mephitis mephitis*) is the primary wildlife rabies vector within North America (2), but has proved refractory to attenuated or inactivated oral rabies vaccine immunization. To test the applicability of recombinant vaccine in this important species, groups of male and female skunks (4 month-old) were inoculated with V-RG virus by several routes: scarification on the flank; oral administration in synthetic baits; duodenal deposition by fiberoptic endoscope; and i.m. inoculation (33). To rule out the development of adverse clinical signs due to vaccination, all animals were observed daily for 3 months. Rabies VNA titers were determined on days 14, 28, 60, and 90 post-vaccination. Only a single skunk seroconverted at 14 days post-ingestion of sponge baits, but by 4 weeks 6/7 skunks consuming the V-RG-bait had demonstrable VNA titers (Table 9). Rabies VNA titers were recorded at 14 days post-vaccination for 5/8 intestinal vaccinates, 4/4 i.m. vaccinates, and 6/6 in the scarification group. Three animals that did not have VNA titers at this time failed to subsequently seroconvert during the 90 day observation period. Not surprisingly, levels of VNA were higher in the animals that were inoculated i.m. or by scarification. VNA titers decreased in all groups over the 3 month observation period, at the end of which all vaccinated and control skunks were challenged i.m. with 0.3 ml of a 10% salivary gland suspension from naturally infected skunks ($10^{6.3}$ $MICLD_{50}$), and observed for 90 days thereafter.

Five of 7 skunks vaccinated by eating bait survived challenge, including 1 animal having no detectable VNA on the day of challenge; 1

Table 9. Induction of VNA in Skunks and Protection from Rabies by V-RG

Inoculation Route[a]	Relative Median Rabies Titers (IU/ml)					Protection[b]
	Day 0	Day 14	Day 28	Day 60	Day 90	
i.d.	<0.1	85.0	8.3	2.5	0.7	5/6
i.m.	<0.1	22.3	12.5	2.6	2.9	2/3
Oral (via sponge baits)	<0.1	<0.1	1.4	0.7	0.2	5/7
Intestinal (via endoscope)	<0.1	0.8	0.5	0.4	0.2	4/8

[a] On day 0, 10^9 PFU V-RG stock was diluted 1:5 in PBS diluent. For i.d. scarification, diluted virus was further mixed 4:1 with glycerol; otherwise, 1.0 ml of diluted V-RG stock was inoculated i.m. while oral and intestinal test group skunks received 5.0 ml of diluted V-RG stock virus.

[b] All skunks were challenged i.m. on day 90 with $10^{6.3}$ $MICLD_{50}$ of a salivary gland suspension from naturally-infected rabid skunks.

(Adapted from ref. 33).

non-rabid skunk died of an apparent bacterial infection, while the other fatality in this group died despite having low level VNA at challenge, bringing the observed survivorship rate to 83% (5/6). Four of 8 skunks in the intestinal group survived challenge, 3/4 in the i.m. group, and 5/6 in the scarification group: all fatalities had low or no detectable VNA at challenge. All controls developed lethal rabies between the 3rd and 4th week post-challenge; non-protected vaccinates mirrored this same incubation period, except for 1 skunk in the scarification group that died on day 70 post-challenge. At no time in these studies did the skunks inoculated or fed V-RG virus-filled bait appear ill. In the scarified group, a few typical vaccinial scabs did form on the cutaneous abrasions as expected, but no pustules were observed. Furthermore, no lesions were detected by gross or histopathological examination of the alimentary tract or visceral organs in any animal fed V-RG laden baits, and sequentially killed over an 11 day period. No rabies viral antigen was detected in the brains of surviving vaccinates when euthanized (33).

Other Wild Species

Oppossum

Whereas safety and efficacy of V-RG in target species is paramount, non-target species have also been examined. For example, the common opossum (*Didelphis virginiana*), while infrequently included among wildlife rabies reporting statistics (2) and considered somewhat resistant to lethal rabies infection, is nonetheless locally abundant and widely distributed throughout North America. Since the opossum has dietary and denning preferences which overlap with those of raccoons and skunks, and is frequently live-trapped in our programs designed to study rabies control and baiting strategies in the former 2 species, it is a potentially important non-target species for consideration in a U.S. vaccine-baiting program. Due to these factors, preliminary safety tests were conducted on 6 captive, individually-housed opossums seronegative for rabies VNA. Each was manually restrained and 1.0 ml ($10^{7.0}$ PFU) of V-RG vaccine was deposited in the oral cavity and the animals allowed to swallow at will. Thereafter, blood was obtained by jugular venipuncture or cardiac puncture for potential V-RG virus isolation upon BHK-21 cell culture and to document development of rabies VNA. No viremia, local lesions, or aberrant clinical signs were noted. By day 30 post-V-RG ingestion, all opossums had demonstrable rabies VNA (GMT = 18.0; range = 0.9-56.8 IU/ml): the animals were not challenged with rabies virus.

Badger

Preliminary safety and efficacy trials of European non-target and domesticated animals have also been communicated (34). In a study conducted by Rhône Mérieux, 6 European badgers (*Meles meles*) received 10^8 PFU of V-RG vaccine via the oral route (by infusion). Two unvaccinated control badgers were kept in close contact with the vaccinates and 2 badgers were maintained as isolated unvaccinated controls. Rabies VNA titers were determined on days 14 and 28 and the animals were challenged i.m. on day 45 post-vaccination with $10^{5.2}$ $MICLD_{50}$ of street rabies virus. Only 2 of the vaccinates developed significant VNA titers and 3 of the 6 animals survived challenge. The results indicate a low "vaccine take" in this species. No transmission was observed in contact controls (P. Desmettre, personal communication).

Wild Boar

In an additional study conducted by Rhône Mérieux, 4 wild boars (*Sus scrofa*), weighing between 12 and 30 kg, were each given a V-RG viral dose of 10^8 PFU orally. The animals were kept with unvaccinated contact-controls. As none of the controls (including 2 unvaccinated isolated animals) died due to peripheral street challenge with rabies virus, protection after challenge could not be measured; only immunized animals demonstrated VNA. There was no indication of either clinical illness or disseminated VV infection in either vaccinated animals or their controls.

Domestic Animal Species

Dog

Preliminary immunization of domestic dogs by s.c. and oral administrations of the V-RG virus was conducted by Rhône Mérieux (34). Evaluation of rabies VNA in groups of 3 dogs inoculated s.c. with serial doses of vaccine indicate that all dogs responded to at least $10^{6.6}$ PFU of V-RG or greater ($10^{8.6}$ PFU), producing 2 logs or better of VNA 14 days after inoculation, and were completely protected against i.m. street rabies challenge on day 69 (P. Desmettre, personal communication). Only 1 of 3 dogs receiving $10^{4.6}$ PFU of V-RG s.c. survived challenge. Dogs receiving these same dilutions by the oral route did not respond as quickly serologically, but did respond well (>2 logs VNA) by 28 days to $10^{9.6}$ PFU per dose. Total vaccine safety was observed, even at the $10^{9.6}$ PFU dose per dog, and 4/4 dogs survived peripheral street rabies virus challenge on day 69 in which all 5 control dogs succumbed. Only 2/4 survived at the $10^{8.6}$ PFU V-RG dose.

Cat

Vaccination of cats by s.c. inoculation or oral administration of V-RG virus also induced rabies VNA, as expected. Following vaccination by the s.c. route with 10^8 PFU, 3/3 cats responded; with 10^6 PFU, 2/3 cats responded; with 10^4 PFU, 0/3 cats responded. Maximum rabies VNA were reached in the responding vaccinates on the 10th day. Protection from challenge by i.m. inoculation (in the neck muscles) with $10^{4.6}$ $MICLD_{50}$ of street rabies virus was total in cats vaccinated with 10^6 and 10^8 PFU of V-RG. Vaccination by the s.c. route produced no adverse

clinical signs. Following administration of V-RG recombinant virus via the oral route with 10^8 PFU, both safety and non-transmission to unvaccinated contact controls was again demonstrated. Two of the 4 cats vaccinated displayed high rabies VNA titers, and both resisted street rabies virus challenge by i.m. inoculation of $10^{4.6}$ $MICLD_{50}$.

Ferret

The same was reported for 2 groups (2 subjects/group) of domestic ferrets (*Mustela putorius furo*), orally immunized with either 10^8 or 10^9 PFU of V-RG and placed in contact with naive cage-mates. Although inoculated ferrets demonstrated the induction of VNA ranging from 1.3-15.3 IU/ml by day 28 post-immunization, naive contact controls did not (P. Desmettre, personal communication).

Cattle

Bovine paralytic rabies of vampire bat origin (primarily *Desmodus rotundus*) results in the death of several million head of cattle in Latin America annually. In a collaborative study conducted by the Pan American Health Organization to determine the safety and efficacy of recombinant vaccine, 10 lactating Holstein cows were inoculated s.c. in the neck with 1 ml of V-RG virus (10^8 PFU per animal) and 10 others were vaccinated by i.d. scarification in a previously shaven area on the neck. Each group of 10 cattle was kept in close confinement with 10 non-vaccinated contact control cows. All vaccinated animals developed significant titers of rabies VNA, while non-vaccinated control animals did not (Table 10). Since control animals remained immunologically naive on the basis of seroconversion, these results strongly suggest (but do not entirely discount) that V-RG virus did not spread between conspecifics. Moreover, none of the animal caretakers demonstrated a rise in rabies-specific VNA. All animals actually receiving vaccine developed high VNA titers by day 15 that were compatible with previous reports of bovine protection against lethal rabies virus infection (35). Except for the typical pox lesions that developed on the neck of the 10 cattle immunized by scarification (and which were confined to the actual site of inoculation), none of the vaccinated cattle showed signs of illness or any decrease in milk production throughout the trial period. Unfortunately, these animals were not challenged with rabies virus (36).

Table 10. Induction of VNA in Cattle by V-RG Vaccine[a]

Inoculation route (n = 10/group)	Rabies VNA GMT (range)	
	Day 0	Day 30
i.d.	<10	1,180 (329-2,701)
i.d. contact controls	<10	<10
s.c.	<10	760 (314-2,701)
s.c. contact controls	<10	<10

[a] Vaccine was inoculated on day 0 using 1.0 ml (10^8 PFU) of V-RG and immunized animals housed with naive contact-control cattle.

(Adapted from ref. 36).

Sheep

In a limited trial, 4 domestic sheep 1-3 yr in age, seronegative against rabies virus, received 1.0 ml ($10^{7.0}$ PFU) of V-RG vaccine deposited directly on the tongue (37). Only 1 of the 4 developed rabies VNA; no buccal lesions, fever, or systemic illness were noted. Insufficient virulence of the rabies challenge virus prevented conclusions regarding protective immunity.

FUTURE PROSPECTS

The efficacy and safety of V-RG are matters of record, whether it is administered: (i) via the intradermal route (mice, rabbits, cattle, foxes, raccoons, skunks); (ii) via the subcutaneous route (rabbits, cats, cattle, foxes, dogs); (iii) via the intramuscular route (mice, rabbits, raccoons, cattle, skunks); (iv) via the oral route (mice, rats, rabbits, cats, dogs, sheep, raccoons, foxes, skunks, ferrets, badgers, opossums, hedgehogs, wild boar; or (v) via the intestinal route (skunks).

The variety of species, inoculation routes, and challenge viruses used (including the rabies-related lyssavirus, Duvenhage), and the resulting immunogenic and protective activity, make V-RG one of the

most versatile rabies biologicals available to date, and a most suitable candidate as a wildlife oral immunogen. A recent review (38) of other VV expression vector systems also includes cotton rats, humans, and chimpanzees in the list of species tested thus far for safety and immunogenicity. Nevertheless, several precautions (39,40) must be weighed before the deliberate environmental release of VV or other (e.g. adenovirus, raccoonpox, fowlpox, herpes, baculovirus, etc.) live recombinant vaccines; most notably, any residual virulence for non-target species (including humans) and the possibility of reservoir establishment arising from recombination with natural poxviruses.

Recent debate has focused upon the selection of one "ideal" VV strain for broadest recombinant vaccine development and application (41). Yet, despite claims to the contrary, it is extremely difficult to compare the relative pathogenicity of VV strains between animal species, and even more dubious to extrapolate retrospective experimental results to humans owing to the lack of an appropriate animal model (42). Indeed, in humans, VV is only marginally pathogenic. Frequency of post-vaccinal complications following its extensive use in the smallpox eradication campaign varied between strains, but also by geography and with the age and immunological status of the host population. Actual virus isolation was not practiced in most cases, but rather a temporal association made with recent VV immunization alone (43). Thus, reasonable estimates of VV inoculation complications (e.g. post-vaccinal encephalitis) are somewhat constrained, since epidemiological studies of VV often do not include a denominator, a failing compounded by poor inter-country surveillance and unreliable case definitions (42).

Theoretically, the expression of foreign surface glycoproteins could result in altered VV tropism, yet the putative association of rabies G with the V-RG virion has not resulted in enhanced neurovirulence, nor has CSF analysis of immunized animals suggested acute or chronic V-RG replication in the CNS (C. Rupprecht and C. Lanutti, unpublished data). Tests of a recombinant VV regarding direct human contact and calculable relative risk would require field trials involving literally millions of human subjects. Transmission of V-RG indirectly to humans through vaccinated animal exposure should be an

exceptionally rare event. Furthermore, V-RG has a TK^- phenotype, a characteristic shown to result in viral attenuation (12). Notably, V-RG does not produce adverse reactions when inoculated into immunodeficient nude mice (M.-P. Kieny, unpublished data; K. Charlton, personal communication), in contrast to a phenomenon described for other recombinant VV (44), which may be particularly mouse-adapted. Similarly, it is scientifically inaccurate to extrapolate the chances of complications arising in immunodeficient patients (45), such as a disseminated infection from a parental VV strain, to a much attenuated vector such as V-RG, where humans are not the primary target.

As an exercise in futility, one can attempt to "guesstimate" the associated risk resulting from a limited field trial with V-RG, making several broad assumptions. For example, if one assumes: a reported historical figure of serious post-vaccinal complications for the parental Copenhagen VV strain as approximately 6 per 100,000 primary vaccinees; an attenuation factor of 1/100 due to TK inactivation of V-RG from its parent; that approximately 70% of raccoons in the field site consume bait and contact vaccine; a 1/100 probability of average human/raccoon "contact" on the study site; and a 1/100 chance that this raccoon has the ability to excrete or transmit virus through tissue contact over its lifetime; then the associated risk of serious complications to human health are somewhere on the order of 10^{-14}, far much less a threat then the acknowledged lethal danger presented by widespread toxicant use in modern rodent control practiced today.

The concern that V-RG would adapt itself to free-ranging wildlife or domestic animals is also remote. Despite its extensive use worldwide, there is no good evidence that VV becomes established in natural animal populations (46), even when considering the close-knit bond and intimate physical contact between animals and humans, and their pivotal role in many rural societies. While some researchers consider *Homo sapiens* as the principal VV host (46), the experimental VV host range probably extends throughout the Class Mammalia. Notwithstanding, there were historical reports of bovine VV cases during peak smallpox vaccine usage, not only from human to cow, but vice-versa, yet reported VV cases in other domesticated species or zoo collections are limited to Europe (almost inevitably connected to contaminated milkers' fingers or

to fresh lesions) or were often confused with other virus infections (47,48,49) (e.g., herpes, paravaccinia, buffalopox, cowpox, etc.) that present with similar clinical manifestations. Bovine cowpox remains extremely rare today, and actually began a decline during the period that VV immunization was most prevalent (50). It must be remembered that VV is an independent stable viral species which has been used for centuries, and given the very real potential for wild-type poxvirus recombination (51), has yielded no clues as to alteration into any agent or syndrome more drastic than itself as an independent viral species (e.g. reversion to variola, etc.) (52,53).

These few but important theoretical risks relating to V-RG field usage are greatly outweighed by the documented benefits accrued through diminution of the acknowledged public health and socio-economic significance associated with international animal rabies. As opposed to other etiological agents, the logistical advantages of a V-RG vector are quite similar to those originally outlined for its choice as a human smallpox vaccine (54), namely: (i) ease of administration (e.g. via the oral route); (ii) potency across broad species range, including the principal wild vectors; (iii) economy of relatively simple, affordable production (e.g. locally if necessary) in cell culture or calf lymph; (iv) stability for months, even at tropical ambient temperatures (especially if lyophilized) without refrigeration; (v) durable humoral and cellular immunity in primary host species with a single, adjuvant-free, administration; (vi) safety, with no adverse clinical signs, gross, or histopathological lesions observed in target or non-target species by the oral route; and (vii) rabies hazard elimination, with no potential of reversion to a more virulent rabies variant as reported for some modified-live rabies virus vaccines.

Taken together, these data strongly support the suggestion that limited field trials with V-RG in an island or other limited ecological setting should proceed. Given the limitations imposed on future laboratory study with animal populations that vary drastically with regard to nutritional plane, parasite load, and environmental stress from their wild counterparts, and the fact that "Noah's Ark" cannot be reliably managed indoors from the realm of species diversity, practicality, and cost, questions relating to safety, efficacy, and feasibil-

ity of V-RG must be tested under restricted field conditions, as were the initial field trials with attenuated wildlife rabies vaccines (3,4). In fact, one such limited trial is under way (55). On 28 October 1987, 250 chicken-head baits containing V-RG vaccine were distributed by hand, on a 2.5 hectare study plot of a secluded Belgian military camp, the Roi Albert de Marche-En-Fammenne, under the direction of Professor P. Pastoret of the University of Liège. Over the next 2 years, surveillance will entail study of the effect of V-RG in both target (e.g. foxes) and non-target (e.g. small mammal) species. In the realm of the global rabies problem, and the potential incorporation of multiple heterologous viral genes into the VV genome (e.g., herpes simplex, vesicular stomatitis, transmissible gastroenteritis, respiratory syncytial, etc.), it is hoped that additional trials may follow suit with V-RG and related VV vaccines for human (56) and veterinary applications.

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16

CONTROL OF WILDLIFE RABIES: EUROPE

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ABSTRACT

In the present rabies epizootic in Central Europe, the chain of infection is maintained largely within fox (*Vulpes vulpes*) populations, with other species being involved only sporadically. With the failure of fox control campaigns in most parts of Europe, investigations into the possibility of immunizing foxes with live attenuated rabies virus administered by the oral route were initiated about 15 years ago. Chicken heads were chosen as baits. A "blister-package" designed to release vaccine (a clone of the SAD strain, grown in BHK-21 cells) into the oral cavity of a fox chewing the bait was developed. The first field trial was conducted in an Alpine valley in Switzerland in 1978, and an advancing epizootic wave was stopped by a barrier consisting of a population of about 60% immune foxes. The strategic application of oral vaccination in additional regions has freed 80% of the country from the zoonosis. In the Federal Republic of Germany, field trials were initiated in 1983. Switching to an automated bait manufacturing system in 1985 allowed an extension of the vaccination areas in Germany, and an export of baits to other European countries, including Italy, Austria, Belgium, France, and Luxembourg.

EPIDEMIOLOGY OF RABIES IN EUROPE

For unknown reasons, rabies disappeared from Central Europe around the turn of the century. Later, during World War II, a new fox rabies epizootic originated in Eastern Europe. Its wave front progressed slowly but continuously towards the west and southwest. The epidemic has been described and analyzed by numerous authors (see Blancou, this

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volume), its main features being as follows:

1. The first rabies cases registered in a newly invaded area are almost always foxes (1,2).
2. The front wave moves into new areas at a rate of approximately 25-60 km/ year (1-6).
3. Rivers, lakes, and high mountains function as natural barriers. Rivers are usually crossed where bridges are available (7).
4. Very intensive fox control may result in areas of low fox density. Such areas may also stop the spread of rabies.
5. The case density in the front wave is very high. In areas with good surveillance, up to 2 rabid foxes per km^2 are recovered yearly (2).
6. Foxes constitute the majority (60-85%) of all diagnosed rabies cases.
7. In animals grouped according to the conditions under which they are collected (shot by hunters, road kills, found dead, killed because of abnormal behavior, etc.), the proportion of rabid ones is always higher in foxes than in similarly grouped categories of other species (2).
8. In an area of a few hundred km^2 the front wave of the epizootic lasts no more than 1-2 years, after which rabies may disappear for several years.
9. In situations where rabies, together with fox control, reduce fox populations below a certain level, rabies disappears not only in foxes, but also in all other terrestrial species. The same observation is made in areas from which rabies disappears as a consequence of oral fox immunization. Only bat rabies is independent of the occurrence of the disease in foxes.
10. Foxes and badgers (*Meles meles*), but no other species, are reduced in population density by the event of a rabies epizootic.
11. An area that becomes free from rabies may be reinvaded after a few years from adjacent infected regions. This is explained by a rapid recovery of fox populations during the rabies-free years (8). The same may occur in areas freed by fox vaccination due to the rapid population turnover bringing the herd immunity below the threshold needed.

12. Of foxes with demonstrable rabies antigen in their brain, 93% also had infectious virus in their salivary glands. This percentage is higher than found in other European wild carnivores (9).
13. None of approximately 1,000 foxes from rabies-infected areas with rabies-negative brains contained any detectable virus in their salivary glands (9).
14. Low titers of rabies-neutralizing activity are found in a small percentage of sera taken from rabies-negative foxes from areas either with rabies or having recently experienced rabies (9,10). Titers may not always be specific, and are usually too low to be interpreted as being the result of survival of clinical disease. The prevalence of neutralizing activity increases to over 50% in areas with oral fox immunization (11).
15. An intensive search for virus and antibody in other species, especially in small mustelids, insectivores, and rodents, has failed to reveal any indication of a rabies reservoir outside foxes (12). Additionally, in these same species, no evidence was obtained that there was any spread of the vaccine virus used for oral fox vaccination (13).
16. The virus circulating in central Europe in foxes is antigenically quite uniform. It is clearly distinct from the virus occurring in bats in northern Europe.

Crucial for the survival of rabies is that the virus is transmitted by an infected fox to enough susceptible individuals during the short period of virus excretion. The rate of infectious contacts is density-dependent. Rabies transmission ceases when population density drops below a certain level or when herd immunity reaches a (density-dependent) threshold. Spread of rabies throughout the countryside probably goes from animal territory to territory, and only rarely over longer distances. It is most likely due to the abnormal territorial behavior of clinically ill foxes coming in conflict with healthy, non-immune individuals, such as when a sick (disoriented) individual intrudes into the territory of a neighbor.

OBJECTIVES AND METHODS OF RABIES CONTROL

Rabies control programs can have quite different goals. The ultimate purpose is always the protection of man from infection and from economic losses. An administration faced with the problem of preventing human rabies has to make decisions on control strategies. The incidence of human rabies may be controlled by elimination or immunization of the domestic animal species or population segment responsible for the transmission to man. Clearly, prophylactic vaccination of persons at risk, treatment of exposed people, and public health systems providing easy access to these treatments are integral parts of any strategy for rabies control. A far more ambitious task is the control of an epidemic in wildlife. Theoretically, this can be attained by drastic population reduction or by mass immunization of the major host species.

Rabies Control by Fox Population Reduction

Three observations demonstrate a clear relationship between the occurrence of rabies and fox population density in Europe:

1. Rabies always disappeared from areas where the disease itself and control efforts had reduced the fox population density to a low level. The drop in population density is usually reflected by a 5-10 fold decrease in the number of foxes shot during regular hunting (12).
2. Rabies did not penetrate into regions with traditional small game (hare, pheasant) hunting (14). In these areas, foxes are considered a pest and are systematically killed wherever and whenever they are found. In other parts of Europe, foxes are killed during the hunting season only.
3. Areas with low carrying capacities for foxes, such as marshlands and high altitude habitats, proved to be barriers not penetrated by rabies (7).

Nevertheless, successful fox control campaigns have remained very rare despite the above observations. In an endemic situation, decimation, in combination with rabies as an important cause of mortality, often succeeds locally in reducing host population densities below a threshold level at which disease transmission ceases. During the subsequent

absence of rabies, however, these populations recover rapidly, and reintroduction of the disease is the consequence.

One method aimed at decimation of fox populations is the fumigation of their dens with toxic gas, and this was widely used in the 1950s and 1960s. Foxes are not bound to specific dens, however; they investigate and use a great variety of different shelters within their home range. In a survey in Canton Berne, Switzerland, we found that the majority of litters are raised in badger earth dens, although natural caves, empty barns, buildings with raised wooden floors, as well as other shelters are also used to hide and raise offspring (8,13). Only dens dug in earth can be gassed effectively, and if this is done in spring a large proportion of the total offspring is destroyed. However, many adult foxes do not rest with their litters, and therefore escape the den gassing campaign.

The surviving animals probably avoid the disturbed den during the next breeding season. This is demonstrated in a shift in the shelter use pattern in areas with den gassing: vixens increasingly use whelping sites that cannot be treated with toxic gas. In one area studied, the number of observed litters remained about the same, but the percentage seen in earth burrows declined to 63% after the first year of gassing, and to 49% after the second (8).

Under pressure from humane societies and public opinion, den gassing programs were largely abandoned in most European countries after 1975. For the same reasons, poison baits are now used only locally.

In most countries of continental Europe foxes are considered game animals. Consequently, their killing is restricted by rules and hunting laws. Trapping, except with live traps, is mostly illegal. Even so, these laws have been relaxed in the face of rabies epizootics, since shooting alone did not reduce population densities below the threshold necessary for stopping the spread of the disease. In an unpublished study of fox hunting in Switzerland it became clear that there is no simple linear relation between the cumulative effort of fox hunters and the percentage of the fox population killed. Different hunting methods are used in different temporal patterns throughout a hunting season. This pattern partly depends on local meteorological conditions, partly

on regional traditions. Hunters grouped according to their professions shoot different numbers of foxes per hunter and season. The application of Leslie's method of estimating population size by comparing the number of foxes killed each week with the cumulative number killed in previous weeks indicates that the fox population becomes larger in the second half (December - February) of the hunting season. This illogical result can be explained on the basis of either decreasing hunting effort and/or decreasing vulnerability of foxes to hunting during the season. Young foxes are easier to shoot than older ones.

The fox's resilience to persecution, and the high reproductive potential in connection with high carrying capacities of rural and suburban habitats, often renders control efforts unavailing. For the red fox, human predation has long been the most important mortality factor other than rabies and sarcoptic mange. The species has adapted well to this situation. Accordingly, there are only a few documented instances where population control measures actually inhibited the spread of an epidemic into a new area. Only Denmark succeeded in creating an artificial barrier of low fox density in South Jutland, thereby protecting the rest of the peninsula (16). Using the same methods, less spectacular results have been obtained in other parts of Europe (8).

Rabies Control by Fox Immunization

The Development of the Method

In several regions all over the world, canine rabies has been eradicated by mass immunization of dogs (17; Larghi *et al.*, this volume). If mass immunization of other vector species were possible, this method would become the most powerful tool in rabies control. But most early attempts to establish reasonable levels of herd immunity in populations of wild carnivores failed (18). A breakthrough came when it was found at the Centers for Disease Control, Atlanta, Georgia, U.S.A., that live attenuated rabies virus immunized foxes by the oral route (19,20). This discovery indicated the possibility of an oral vaccine which could be administered by bait, and stimulated European rabies research teams to work toward fox rabies control by oral immunization. However, there was still a series of vaccine- and bait-related problems to solve. It was necessary to find a safe and potent vaccine for field

application, and a vaccine delivery system assuring immunization of the target species.

In the early 1970s, laboratory studies and related epidemiological and ecological field investigations were performed in Europe at the National Centre for Studies on Rabies in Malzéville, France; at the State Veterinary Research Institute in Frankfurt, Federal Republic of Germany; and at the Institutes of Veterinary Microbiology and Zoology, University of Berne, Switzerland. The World Health Organization coordinated the research efforts and organized conferences. In addition, we Europeans received virus strains, information and moral support from Centers for Disease Control, Atlanta, the New York State Health Department Laboratory in Albany, New York, U.S.A., and the Wildlife Research Division of the Ministry of Natural Resources in Maple, Ontario, Canada.

Oral Vaccine Administration and Immunogenicity

Oral immunization with a rhabdovirus bears some handicaps. Rabies virus is sensitive to acids and it loses its infectivity in the stomach. Therefore an unprotected live-attenuated rabies vaccine needs to infect oral or/and pharyngeal mucosal tissues in order to elicit an immune response (21). Animals may also be immunized by inoculation of live attenuated vaccine directly into the small intestine (37). A vaccine vehicle which could be swallowed and which would release the vaccine after passing through the stomach would provide advantages over the oral/pharyngeal mode of immunization. In the intestine, the contact of the vaccine with host mucosa would be more certain than during the brief passage through the oral cavity. The problem of protection of the vaccine from degradation may be solved with a protective coat around a pellet of lyophilized vaccine, or with vaccine absorbed onto a carrier. The protective mechanism would have to resist humidity, low pH, enzymes and elevated temperature before releasing infective virus into the mildly alkaline milieu of the small intestine. Unfortunately, however, all attempts to find a simple, inexpensive, safe, and effective procedure have failed so far.

The first demonstration that foxes can be immunized by the oral and by other non-parenteral routes against rabies was made with the SAD strain, and later with its derivative, ERA (19,20,22-24). (See Bunn,

this volume, for details on these strains). All foxes developed neutralizing antibodies when liquid vaccine containing at least $10^{4.5}$ $TCID_{50}$ (50% tissue culture infectious doses) of SAD per dose was given directly into the mouth cavity. When 1-2 ml was included in a bait, the virus content needed to be above 10^6 $TCID_{50}$. All animals showing seroconversion resisted challenge when tested (18,25,26-30). The high titers required are easily produced in tissue culture, and the vaccine is therefore relatively inexpensive. It is also quite stable and easy to store. In the Swiss field trials, 10% egg yolk is added to the virus stock as a stabilizer.

Both other widely known attenuated rabies strains are not suitable. LEP-Flury is too pathogenic for most species (and probably also for foxes). HEP-Flury has a much lower pathogenicity (31,32), but it is not sufficiently efficacious. HEP also easily reverts to a virus with higher pathogenicity (33). A few additional mutants of different rabies strains have been tested for immunogenicity and innocuity (34,35), but so far none of them have proved to be superior to SAD. Immunization with inactivated vaccine by the intestinal route has also received some attention. Highly concentrated antigen needs to be brought intact through the stomach into the small intestine, and a series of booster doses is usually required before the animal mounts a detectable immune response (36-39). Oral and intestinal immunization of wild animals with killed vaccine is clearly not the method of choice, at least until new technologies to allow an efficient transfer of swallowed antigens through mucous membranes to immunocompetent cells.

Some of the problems of live attenuated vaccines may be overcome when recombinant viruses expressing rabies glycoprotein are used. A derivative of vaccinia has been applied with great success in trials to immunize a variety of mammalian species by different routes (40-45; see also Rupprecht and Kieny, this volume). Foxes, raccoons, and skunks receiving the recombinant vaccinia by the oral route develop neutralizing antibody. But replacement of the presently applied attenuated virus SAD by a live genetically engineered vaccine does not remove all hazards, and this new approach will need intensive further investigation.

Innocuity Studies with SAD Virus

Safety was of prime concern to the participants of the WHO/FAO co-ordinated research programme on oral vaccination of foxes against rabies in Europe. In several laboratories foxes, stone martens (*Martes foina*), weasels (*Mustela erminea* and *M. nivalis*), wild boar (*Sus scrofa*), cats and dogs were given orally varying doses of SAD. No adverse effects were noted, but it has been noted that SAD and other attenuated rabies viruses will kill an occasional domestic animal having an impaired immune response (46). It seemed very likely that the same would happen to a free-ranging carnivore suffering from some immunological deficiency.

The SAD strain has some residual pathogenicity for a variety of rodents (47-49). All important myomorph rodent species indigenous to Central Europe were therefore tested. SAD-induced mortality in these rodents is dose-dependent, is higher in younger animals, but displays a great variation. There is also some indication that there might be differences in pathogenicity among different strains of SAD, or of virus grown in different types of cells, but these differences are not well documented, and may also reflect different inoculation and assay techniques in different laboratories. In rodents dying from SAD-induced rabies, virus can be recovered regularly from brain, and irregularly from brown fat and salivary glands. The virus yield from the brains of adult mice is usually below $10^5 TCID_{50}/g$, but is considerably higher from inoculated suckling mice. Serial oral passage is possible only in newborn rodents. Rodents surviving oral exposure to SAD virus only rarely develop immunity to rabies. These laboratory results indicate that spontaneous SAD transmission from rodent to rodent is a rare event. Additionally, a field trial on a river island in Switzerland, using baits containing SAD designed to be picked up by rodents and insectivores, yielded no indication that SAD became established in the small mammal community (13).

Besides being immunogenic by the oral route for the target species, and being apathogenic for man, target species and other species picking up bait, the vaccine should comply with a number of additional requirements:

1. It should not be excreted. In order to immunize orally, the virus

has to infect tissues in the buccal cavity or pharynx. Some virus excretion is, therefore, to be expected, even if this has not yet been demonstrated when looked for.

2. It should not easily revert to higher pathogenicity. Two SAD strains propagated orally over 20 passages in newborn mice at the Swiss Rabies Centre did not change antigenic, pathogenic and immunogenic properties.
3. It should be free from pathogenic contamination.
4. It should bear at least one genetic marker. Today SAD virus can be identified quite easily with monoclonal antibodies (50).

Baits

Baits serving as vehicles for the vaccine must, in their turn, meet a set of requirements: they must be attractive for foxes, which should eat them without storing; they should be rejected by other species (including man); and they must not inactivate the vaccine, but deliver it into the mouth cavity of an animal picking it up.

The acceptance of a great number of different bait types (without vaccine) was studied in field experiments, and they were tested with vaccine in the laboratory. All bait types examined under European field conditions were also eaten by various domestic and wild carnivores and often also by rodents (51). All of them also inactivated the unprotected vaccine virus with the exception of chicken eggs, as suggested by Debbie (25). However, although chicken eggs are well picked up by foxes, they are then stored for prolonged periods, making them unsuitable for the purpose. The problem of inactivation in meat and tallow baits could be solved by including the bait into a container which is ruptured during bait uptake. This approach, however, creates another problem: foxes often reject a vaccine container incorporated into otherwise relatively homogeneous bait. Considerable effort was therefore necessary for the development of a container delivering the vaccine into the mouth cavity before it is swallowed or rejected (30).

Application of the Method

The Initial Field Trials in Switzerland

By 1978 we had a system we felt would work. Chicken heads were chosen as baits. Cloned SAD with a minimum of 10^7TCID/ml was dispensed

in 1.8 ml aliquots into small plastic blister-packages (30). These vaccine containers were heat-sealed and fixed between the brain case and skin of slaughterhouse chicken heads. As a biological marker, 150 mg of chlortetracycline was injected into each chicken head. Franz Steck, leading the Swiss team until his untimely death in 1982, decided at this point that a field experiment was necessary. The first distribution of vaccine baits in the field was carried out in October 1978 (11,52). The Rhône Valley in the Swiss Alps was threatened by an advancing front of fox rabies, reaching the lower part of Canton Valais in the summer of 1978. A total of 4,050 vaccine baits were deposited in an area of 335 km^2 in the region of Martigny, at the valley entrance. Rabies did not cross the barrier consisting of a population of about 60% immune foxes. The valley above the "immune barrier" remained rabies free. In order to maintain a herd immunity sufficient to inhibit the spread of rabies, vaccination campaigns were repeated in both spring and autumn of the following years. Since there is no direct proof that the rabies spread was stopped by the presence of immune individuals, the experiment had to be repeated in other similar situations, where an "immune barrier" could be built in the expected passage of an epizootic wave front. In no instance was the barrier crossed by the epizootic.

Safety Aspects

In order to minimize the number of undesired contacts of man with the live vaccine SAD it was decided that:

1. All bait handling was to be done with gloves;
2. People handling the vaccine containers during bait preparation were to be prophylactically vaccinated;
3. In the field, baits were to be placed so that the chances of pick-ups by passers-by (children) and domestic animals were minimized;
4. People coming into serious contact with the vaccine (via wounds, mucosa) would be treated as if SAD were a street rabies virus, and would receive post-exposure vaccination.

From 1978 to 1982 all rabies isolates made in baiting areas were checked for their growth properties in tissue culture. Since 1982, all rabies isolates from anywhere in Switzerland have been analyzed with a panel of monoclonal antibodies. All except 3 isolates were identical

to the street virus circulating in foxes. The 3 isolates were indistinguishable from SAD, and originated from animals (1 cat, 1 stone marten, 1 fox cub) behaving abnormally in areas where vaccine baits had been distributed a few weeks before. Two of the isolates were given orally and injected into dogs and cats. The inoculated animals did not demonstrate any clinical signs of rabies, but developed antibody.

European Fox Vaccination Campaigns

Between 1978 and 1982 the repetition of field trials in Alpine valleys freed large areas within the Swiss Alps from rabies. In 1982 a concept for the eradication of the disease in Switzerland was developed. Since high mountain ranges impeding the spread of the disease greatly facilitated the strategic application of fox vaccination, the concept of natural and artificial barriers to impede the spread of disease was also applied to the rest of the country. We divided the country into epidemiological compartments delineated by natural and artificial obstacles to the spread of rabies. Compartment after compartment was treated twice yearly with 12-15 baits /km^2. Most baits were placed by hand in predetermined places in order to achieve an even distribution and to reduce the chances that passers-by might pick them up. In some Alpine areas with reduced accessibility, additional baits were dropped from helicopters. Vaccine baiting was discontinued in a compartment when the disease had disappeared from it. Today the country is free from rabies, except for the non-treated areas bordering highly endemic zones abroad.

In the Federal Republic of Germany the WHO Collaborating Centre at the Federal Research Institute for Animal Virus Diseases in Tübingen started field trials in 1983 (53-56). Switching from the chicken head bait to a machine-manufactured bait in 1985 allowed an extension of the field trails in Germany and an export of baits to other European countries. In northern Italy a test area was treated for the first time in 1984. Since then, Italy has become free from rabies. In Austria the Federal Province of Vorarlberg successfully eradicated rabies by oral immunization campaigns in 1986. Belgium, Luxembourg and France joined in a common field trial in 1986.

CONCLUDING REMARKS

When rabies reappeared in Western Europe after World War II it seemed apparent that the epizootic would be brought under control by decimation of its main vector, the red fox. It soon became apparent, however, that an effective reduction of fox population densities is an extremely difficult task. More and more foxes escaped the destruction campaigns and the general public increasingly objected to these veterinary police measures. To either accept wildlife rabies as an unchangeable fact, or to battle it with an alternative method were the only logical alternatives. The development of a system for the immunization of free-ranging red foxes became a success, thanks to the joint efforts of many European research teams and governments, and thanks to the essential contribution of North American laboratories. There were still a series of vaccine and bait-related problems to solve when respective investigations were initiated in Europe. Several attenuated viruses were tested in laboratory experiments and numerous possible baits became evaluated under field conditions. It was found that the SAD strain fulfilled the majority of the requirements for areas with predominantly fox rabies. The first field trial in 1978 in Switzerland was successful, so that it was followed by additional campaigns. These rapidly freed the major part of Switzerland and large areas of other European countries.

The most important conclusions from these first field applications of vaccine baits in Europe can be summarized as follows. It is possible to immunize enough free-ranging foxes by bait to stop the spread of the disease into rabies-free areas, and it is also possible to eradicate the disease from an enzootic area. Rabies disappears when a threshold herd immunity in the fox population is reached. In areas where this goal was achieved, between 50% and 90% of all foxes were immunized. These figures are based on the demonstration of antibody in serum, and tetracycline in bone, of killed foxes. In areas freed from fox rabies, rabies also disappeared from all other species (except bats). This indicates that the red fox is indeed the only species responsible for maintenance and spread of the rabies strain presently predominant in Central Europe. The disease does not reappear from "below ground" after oral immunization of foxes is discontinued and the

herd immunity drops, and so rabies may reinvade a fox population becoming non-immune. In Europe at least, there are no unrecognized reservoir species such as small mustelids, as is suspected in some other geographic areas.

The success of the present fox vaccination campaigns with SAD virus should not impede further efforts to improve methods of rabies control. We need other approaches for the oral immunization of other carnivores. The residual pathogenicity of SAD is a considerable handicap. Inactivated or genetically-engineered vaccines may provide an answer: the very promising results with recombinant vaccinia virus need careful consideration. Baiting technologies may also benefit from research. Substances attracting specific carnivores and repelling other animals (and man) could help to improve the campaign efficiency and reduce possible side effects.

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17

CONTROL OF WILDLIFE RABIES: THE AMERICAS

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INTRODUCTION

The goal of rabies control in North America is to eliminate human exposures to the disease. There are different levels at which this can be accomplished. The first line of defense involves vaccination of dogs and cats plus control of wandering dogs. However, rabies is enzootic in wild animals in much of the Americas, so complete control must ultimately address elimination of the disease from the wild. That achievement is still in the future, but significant strides have been made towards reducing the major wildlife enzootics within North America, and we may expect dramatic reduction of rabies among terrestrial mammals by the year 2000.

Human exposures to rabies in tropical parts of the Americas originate mostly from dogs (1). However, bat-borne rabies is the largest single disease problem for the cattle industry in the region, and major efforts have been made to reduce the losses to that source (2,3).

HOSTS AND STRAINS OF WILDLIFE RABIES

Application of monoclonal antibody panels to virus isolated from wild animals has dramatically increased our understanding of the status of wildlife rabies in Canada and the U.S.A. An important conclusion has emerged, that each strain of virus is spread in the wild by only a few species. Each has the potential to infect many or all mammalian species, but in different geographical areas appears to be transmitted widely by only 1 or 2 vectors (Blancou; Smith and Baer, this volume).

J.B. Campbell, K.M. Charlton (eds.), RABIES.

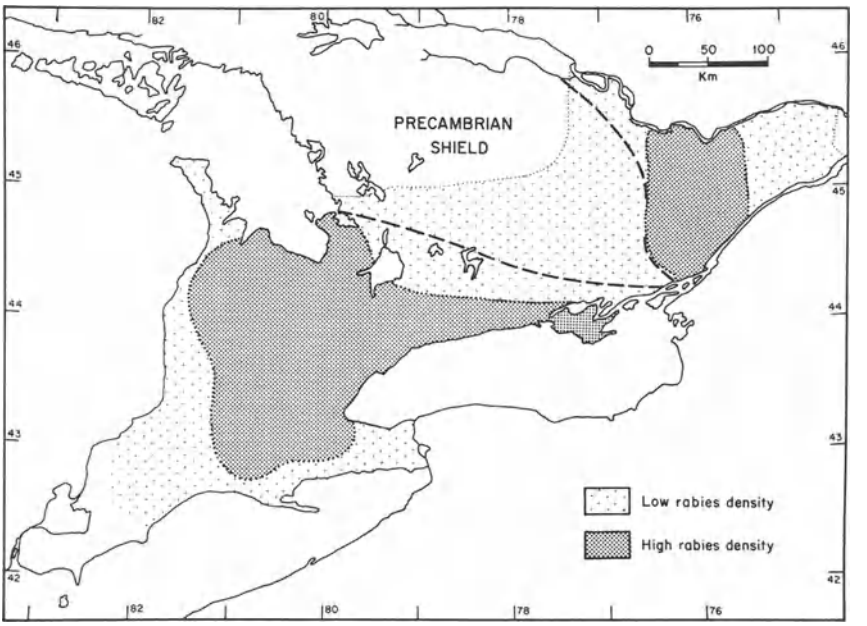

Figure 1. The distribution of rabies in southern Ontario, 1986. The low density of cases along the northern edge coincides with the southern boundary of the Precambrian Shield characterized by extensive forest and little agriculture. The majority of rabies cases occur in better farmland. For details of distribution of fox and skunk rabies, see Tinline (this volume).

Unfortunately, dog rabies still dominates the case statistics from Mexico to southern South America to such an extent that there is no useful information about transmission of rabies among terrestrial wild mammals in that large area (Smith and Baer, this volume).

Rabies in Foxes

The highest density of reported animal rabies in North America occurs in the agricultural lands of southern Ontario (Fig. 1). Most wild animal cases are in red foxes (*Vulpes vulpes*), but striped skunks (*Mephitis mephitis*) are also important (Table 1). There is no evidence that rabies among domestic animals is acquired from any source other than foxes or skunks. The Ontario enzootic extends into northern New York state (across the St. Lawrence River from Ontario) and to a strip of Quebec lying across the Ottawa River. This strain of rabies originated from arctic foxes (*Alopex lagopus*) in the mid-1950s (4).

Table 1a. Domestic Animal Rabies Cases Diagnosed in Ontario, 1958-1986 (Source, Agriculture Canada Monthly Reports)

Species	Number	Percentage of: Domestic cases	Total cases
Cow	7,567	55	18
Dog	2,189	16	5
Cat	1,988	14	5
Sheep	834	6	2
Horse	698	5	2
Pig	402	3	1
Goat	161	1	-
Rabbit	8	-	-
Bison*	4	-	-
Donkey	2	-	-
Elk*	1	-	-
Total	13,854	100	34

* All cases from captive animals. Both these species are very rare in the wild in Ontario and localized outside the enzootic zone.

Table 1b. Wild Animal Rabies Cases Diagnosed in Ontario, 1958-1986 (Source, Agriculture Canada Monthly Reports)

Species	Number	Percentage of: Wildlife cases	Total cases
Red fox	18,519	67	45
Striped skunk	7,939	29	19
Bat*	455	2	1
Coyote/ wolf* *Canis latrans/ Canis lupus*	258	1	-
Raccoon, *Procyon lotor*	245	1	-
Woodchuck, *Marmota monax*	42	-	-
Deer, *Odocoileus virginianus*	8	-	-
Black bear, *Ursus americanus*	5	-	-
Muskrat, *Ondatra zibethicus*	4	-	-
Fisher, *Martes pennanti*	2	-	-
Squirrel, *Sciurus carolinenses*	1	-	-
Weasel*	1	-	-
Vole, *Microtus pennsylvanicus*	1	-	-
Mink, *Mustela rison*	1	-	-
Otter, *Lontra canadensis*	1	-	-
Total	27,482	100	66

* Not identified to species in case reports.
More than 95% of identified rabid bats are *Eptesicus fuscus*.

Another outbreak of rabies occurred in gray (*Urocyon cinereo-argenteus*) and red foxes in the Appalachian mountains of the eastern U.S.A. At one time that form extended from southern New York (5) to the coastal plain in Alabama and Florida (6,7). The area affected by this strain diminished dramatically during the 1970s, and samples are not available for inclusion in monoclonal antibody analyses (S. Jenkins, personal communication). Rabies is also present in gray foxes in Arizona, but no relationship to the Appalachian outbreak is known (Smith and Baer, this volume).

Rabies in Skunks

Skunks have been the recognized source of rabies in the valleys of the Mississippi River and its major tributaries since the early days of European settlement in North America. There are 2 long-standing foci, 1 in Minnesota and Iowa, and another in Texas. Through the 1970s and 1980s, these 2 foci expanded until they met, and skunk rabies was, by 1987, very widespread (8). According to monoclonal antibody analysis, each focus harbors a distinct form of virus (8). There is also skunk rabies in northern California (9), of the same strain as the Minnesota - Iowa focus (8).

Rabies in Raccoons

Rabies spread by raccoons was first noted in Florida in the 1950s (7,10). A new epizootic began in 1979 on the Virginia - West Virginia border, and spread northward through Maryland into Pennsylvania by 1985 (11,13). That outbreak has stimulated great interest in control measures because it has produced many animal rabies cases in urban environments (12).

Rabies in Bats

North America

Rabies has been diagnosed in virtually all North American bat species, but it is not clear whether all can spread the disease (see Smith and Baer, this volume). Migratory bats occupy most of North America south of the northern limit of trees, and rabies has been detected virtually throughout that range.

At least 5 variants of rabies virus have been isolated from these animals (8). Nevertheless, although individual terrestrial mammals can

be infected by bat rabies, none of these strains appears to produce self-sustaining enzootics in terrestrial species (8,13,14). This subject is still controversial, however, because small, isolated outbreaks of rabies have occurred in skunks in Montana, Wyoming (15) and Alberta (16), hundreds of kilometers from the nearest skunk enzootic. One proposed explanation for these is transmission from migratory bats (16), although this has not been confirmed by monoclonal antibody analysis.

Central and South America

Vampire bats, chiefly *Desmodus rotundus*, frequently carry rabies in the American tropics. Losses of cattle to vampire-vectored rabies are serious from Mexico to northern Argentina. Economic losses exceed US$50 million annually (17,18).

There is little information on rabies in other bat species in the American tropics. Insectivorous bats have been confirmed to have rabies, but the position of those species with respect to epidemiology has not been established (see Smith and Baer, this volume).

Rabies in Mongooses

Rabies is enzootic on Granada (19) and other Caribbean islands (20; Smith and Baer, this volume), among mongooses (*Herpestes auropunctatus*) introduced from India to combat rats in sugar cane. Everard and Everard's studies on Grenada present some of the best information from anywhere on the dynamics of rabies in a wild system (19). The origin of the rabies is uncertain.

RABIES CONTROL BY POPULATION REDUCTION

Terrestrial Mammals

Rabies in wild mammal populations fluctuates, probably as a function of density of the principal vector. Therefore, mass killing of vectors to reduce their density has always been an attractive possibility for controlling the disease. In theory it should be possible to depress the host density to a level at which rabies will die out. Nevertheless, the North American experience with vector population control to eradicate rabies has not been encouraging.

The major difficulty in evaluating the publications concerned with population reduction is that none of the studies was designed as a scientific experiment. As Romesburg (21) and Macnab (22) so correctly decried, until that is done we will not understand the reasons for failure. In the terms of Fraser (23), rabies control projects, in common with many other wildlife-oriented programs, have had a beginning and an end, but no decision points in the middle. Despite well-organized logistics, no North American population reduction program began with knowledge of the density of rabies vectors, nor of the reduction required to affect the rabies situation. Davis (24) has pointed out that it seemed important to appear proactive, and the obviously effective alternative, habitat alteration, seemed logistically impossible.

The key difficulty in the absence of experimental design is the lack of experimental controls. Indeed, it would be impossible politically to have an untreated area as part of a rabies eradication program. However, one must evaluate all claims of success against the question of what might have happened if there had been no attempt at control.

A second difficulty arises from severe deficiencies in our understanding of the progression and persistence of epizootic rabies. Rabies generally does not occur throughout the geographic range of its principal vector species. Thus, for example, both red foxes and skunks are found in parts of Ontario well to the north of the enzootic zone. The invasion of rabies from the Arctic passed through northeastern Ontario, but rabies died down there in 10 years, and disappeared completely after only 17 years (Fig. 2).

Knowledge of the dynamics of enzootic rabies is also imperfect. That deficiency was captured by Voigt and Tinline (25): "The fox density at which rabies is epizootic in Ontario is the density at which Europeans claim rabies will disappear" (26,27). The occurrence of 1 or 2 rabid animals at irregular intervals as rabies is dying out is particularly hard to explain under current dynamic models. For example, in northern Ontario there are 2 well-separated agricultural areas, 1 in the District of Cochrane, and the other in the District of Temiskaming between Kirkland Lake and Cobalt, about 100 km to the south. The

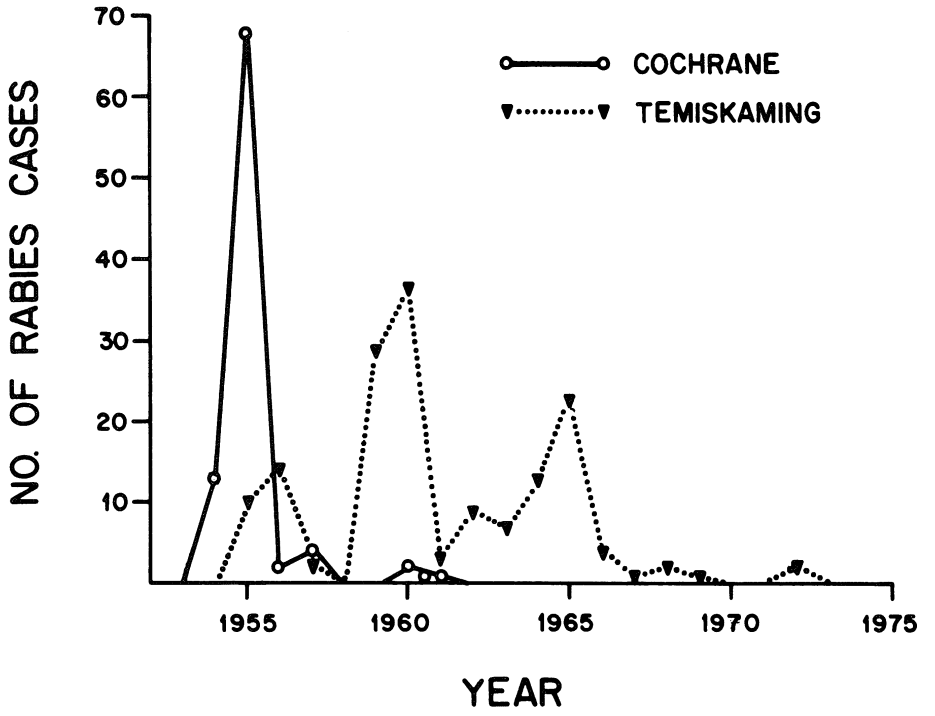

Figure 2. The numbers of reported rabies cases in the territorial districts of Cochrane and Temiskaming in northern Ontario. This illustrates passage of the epizootic which resulted in the arrival of wildlife rabies in southern Ontario.

progress of rabies in those 2 areas is shown in Fig. 2. Even understanding that there are deficiencies in the reporting system (28), it is hard to explain the persistence of rabies at such low levels for 7 years after the major outbreaks. That phenomenon has also been observed in Europe (29). Persistence at such low levels that no cases are reported for up to 2 years also provides a caution to acceptance of claims of successful elimination of rabies by host population reduction. Imagine that the 2 areas shown in Fig. 2 had been subject to control programs stimulated by the initial outbreaks. The rapid disappearance of the disease could readily be interpreted as due to a successful control program.

How much of a fox population must be removed? The answer to that question requires understanding of the reproductive capacity and normal rates and causes of death in the absence of rabies. Various authors (24,25,30,31) report that red foxes can replace 60-70% losses in a

single breeding season. The extent to which death due to rabies adds to the effects of population reduction is poorly understood.

Rabies appeared in wild foxes in southern New York in 1943-45 (32). The basic approach to control involved strategically-located salaried trappers (32,33). Containment zones 16 to 30 km wide were tried, to prevent further spread of the disease. There were some temporary successes, followed by outbreaks beyond the barriers. Concentrated trapping, with some additional gassing of dens, did appear to reduce rabies incidence within the treated area, but by 1965 it became evident to the control team that rabies-induced reduction of fox density might be as great an influence as the trapping program. After almost 20 years of effort, Parks (33) concluded that the program had produced no lasting effect. However, sometime between 1965 and 1975 fox rabies disappeared from southern New York, and the only rabies cases seen there now appear to be of bat origin (34). Outbreaks of sarcoptic mange were noted during the period when rabies disappeared (33,35). That condition is lethal to red foxes, and might, in conjunction with rabies, have helped reduce fox populations to a level below that critical for the passage of rabies, although there is no direct evidence for this (33,36).

The invasion of arctic fox rabies into the Canadian prairie provinces in 1951 was met by an aggressive poisoning campaign mounted by the government of Alberta from June 1952 to March 1954 (37). The 'conservative' estimate by Ballantyne and O'Donoghue (37) suggested that 50,000 foxes, 35,000 coyotes and almost 15,000 individuals of other species were killed in that province, although recent workers doubt that the kill was that high. Rabies persisted through 1955, but then died out, except for 1 isolated outbreak in 1958 (4). However, rabies disappeared at the same time from Manitoba, Saskatchewan and British Columbia, despite the absence of control measures. To be fair, however, there were many more cases in Alberta during the peak years 1952-1955 than in the other jurisdictions, although the active countermeasures in Alberta could have stimulated the local reporting system. The fact remains that it cannot be proven that rabies would have persisted in the absence of the control program, or that the poisoning of potential vectors hastened the disappearance of the disease.

The efficacy of programs to control skunks in enzootic situations has proven equally inconclusive. An intense poisoning and den-gassing campaign covering county-sized areas in Ohio reduced the number of rabies cases below predicted levels. However, the effect lasted only a single season, and the program was deemed too expensive to continue, much less expand (38).

The attempt to keep an invasion of rabies in skunks from entering Alberta is more controversial. Skunks within a zone approximately 30 km wide and 600 km long (39) along the Alberta - Saskatchewan border were subject to stringent control by poisoning, trapping and shooting. This was successful to the point that no rabies cases were recorded among terrestrial mammals within Alberta from 1971 to 1979 (39,40). The program was aided by the fact that skunks in Alberta spend several months in winter dens. Those dens are mostly located near buildings, and contain up to 30 individual skunks (41). Thus the animals were probably more vulnerable to control efforts than most skunks living under more favorable conditions in the U.S.A.

In 1979, 2 large pockets of skunk rabies appeared more than 100 km west of the border population reduction zone (BPRZ). Control was instituted in the outbreak areas, and rabies dropped significantly, but seemed to persist at low levels (40). The route by which rabies reached those 2 pockets is unclear. Rabid skunks may have moved north out of Montana from starting points west of the BPRZ (J.R. Gunson, personal communication). The isolated nature of the pockets is also puzzling: there were skunks in the surrounding country (40), but the densities were not determined with sufficient precision to allow comparison. Rabies levels appeared to be much greater in Saskatchewan (41), so these outlying pockets may represent an extreme extension of the range of rabies. If that were true, rabies should be more vulnerable to control measures, but, equally, the probability seems high that the disease would die out due to natural changes in skunk density or rabies prevalence. Thus, despite the correlation in time between reduction of rabies incidence and proactive vector control, a cause and effect cannot be established. Isolated local rabies outbreaks involving skunks appear sporadically and die out within a few years in Wyoming, in a manner similar to the Alberta situation (15). In fact, a recent

report from Alberta listed 2 rabid cats taken at least 240 km from the nearest recent case of terrestrial rabies, yet the virus was identified as being of skunk origin by monoclonal antibodies (42). The population reduction program in Alberta was cancelled in early 1986, and was soon followed by a modest increase in rabies (R. Rosatte, personal communication). This demonstrated that sustained population reduction had not eliminated the disease, even though there had been no cases for 2 years.

Vampire Bats

The techniques for substantial reduction or elimination of vampire bats appear well developed. Vampire bats are highly susceptible to the effects of anticoagulant drugs (43,44). There are 2 basic tactics for mass treatment of vampires. The anticoagulant may be injected into cattle: the dose does not harm the cow, but effects reduction in vampire attacks by 97-100 percent (44). Two substances, diphacinone and warfarin have been tested successfully (44,45).

In cases where the vampires are feeding on humans, bats may be mist-netted and smeared with anticoagulant (chlorophacinone, diphenadione or warfarin) mixed with vaseline (as a sticky carrier). Upon release, the bats will return to their communal roosts. Since the colony members groom each other extensively, most individuals will ingest a lethal dose (46). This method has drawbacks, however, because vampires may share their roosts with other bat species, so non-targets may also be killed (47), although there are few direct observations of that.

An advancing front of vampire-borne rabies in northern Argentina was halted by gassing roosts (48). That campaign was aided by the fact that all known vampire roosts were in man-dug water wells. Indeed, the whole rabies outbreak may have been man-made, if the digging of wells and ranching of cattle allowed vampire bats to invade country that they were previously unable to occupy.

RABIES CONTROL BY VACCINATION OF WILDLIFE

Since 1968, the emphasis for control of North American wildlife rabies has shifted toward vaccination of the principal vectors (49-53). Plans have, from the first, centered upon vaccination by the oral

route so that the vaccine could be delivered in baits. Five subject areas have received concentrated attention: (i) finding an effective oral vaccine; (ii) proving the safety of the vaccine for humans and non-target species; (iii) developing a suitable bait; (iv) finding the right strategy and tactics for distributing baits; and (v) gaining sufficient understanding of the vector ecosytems to determine the parameters for successful elimination of rabies.

The Vaccines

Modified Live Vaccines

In the early 1970s the most effective rabies vaccines for domestic animals consisted of modified live virus (MLV) (see Bunn, this volume). Rabies virus normally enters the body by a bite wound, and the vaccines simulated this by being administered by injection. However, the demonstration that rabies virus could also be absorbed in an immunogenic form through the oropharyngeal mucosa (54-56) was a critical step towards proving the potential of the oral route for vaccination. Progress in the search for a useful oral vaccine has been reviewed by Baer (53). Several studies have demonstrated the efficacy of various MLV vaccine preparations administered orally to foxes (53,57,58). Skunks have so far proven refractory to MLV in the mouth, although limited and inconsistent success was obtained when vaccine was introduced directly into the duodenum (60-62). Raccoons are not immunized in sufficient numbers by mouth with MLV (71), although there has been limited success in laboratory studies with both the SAD-B19 (C. Rupprecht, personal communication) and ERA/BHK-21 strains (K. Lawson, personal communication).

Killed Vaccines

The perceived safety problems (see next section) associated with MLV rabies vaccines long delayed their use in the field. Effective killed virus (KV) vaccines appeared on the veterinary market in the 1970s, and by 1985 MLV vaccines were mostly phased out of use in Canada. Therefore, tests were conducted to determine the applicability of KV vaccines to immunization of wildlife. Some success was achieved with domestic animals (cats (63) and mice (64,65), especially when the oral doses were adjuvanted with *Quillaja* saponins (66)). Target wild species responded well immunologically to parenterally-administered KV

vaccine, but no wild species has reacted well enough to provide the basis for mass immunization in the field. Brochier and coworkers (67) obtained seroconversion in 6/6 red fox cubs fed 10 daily doses of a commercial KV rabies vaccine (Rabisin; Rhône Mérieux); however, the antibody responses lasted less than 28 days, and the subjects died on challenge. Lawson (personal communication) was unable to obtain seroconversion in foxes after oral administration of KV ERA vaccines, but Rupprecht (personal communication) achieved high antibody titers in up to two-thirds of raccoons given a highly concentrated and purified ERA-derived KV vaccine.

Limited success was achieved when KV vaccine was administered directly into the duodenum of red foxes by gastroscope or other means (68-70). So far, however, it has not been found possible to raise the seroconversion rate above 30% of individuals treated, even after a booster dose.

Recombinant Vaccines

The development of a recombinant DNA rabies vaccine (V-RG), using vaccinia as the carrier virus expressing the rabies G protein as part of its surface, has stimulated much excitement among those working towards large-scale vaccination of wildlife in the field. The preparation is highly immunogenic in raccoons (71), skunks (61) and foxes (72), as well as all other species on which it has been tested (see Rupprecht and Kieny, this volume). The virus can successfully enter a target animal by a wide range of routes (71), and so far appears to be stable for several days under field conditions. The perceived safety risks appear lower than those associated with MLV rabies preparations, although there is no way to make a direct comparison of the comparative dangers to humans who accidentally contact the vaccine. Other recombinant systems are under consideration, including use of canine adenovirus (73, and J. Campbell, personal communication) and raccoon pox (G. Baer, personal communication) as the carrier virus.

Recombinant vaccines appear to be the preferred vehicles for future wildlife vaccination programs. They are more consistently immunogenic against all the important wild rabies vector species, and appear safer and more stable under field conditions.

Safety Considerations

MLV rabies vaccines can cause clinical rabies in a variety of animal species. Mice are particularly susceptible (60,68,74), and there has been concern that field use of MLV vaccine could trigger an epizootic of rabies in rodents (75). However, recent studies have clearly indicated that viruses isolated from cases of vaccine-induced rabies remained vaccine strains (76), and that they are unlikely to be transmitted mouse-to-mouse. Foxes fed mice paralyzed by ERA vaccine seroconverted (68), and 3/6 resisted challenge with street virus. Cats fed similarly showed no serological reaction (K. Lawson, personal communication).

Large scale use of a high-titered MLV vaccine in the field in Europe has produced very few cases of vaccine-induced rabies (77; Wandeler, this volume), and no evidence whatsoever for spread of vaccine virus from animal to animal in the field (60).

Human safety is also at risk. There has been at least 1 laboratory accident in which a high-titered MLV rabies vaccine was implicated (78). Use of vaccinia in the successful world-wide campaign to eliminate human smallpox provided hundreds of millions of tests of the safety to humans of the parent of V-RG. Vaccinia did cause some medical complications, but in very low frequency (79). The most extreme problems included widespread pox lesions and post-vaccinal encephalitis, which was sometimes fatal (80,81). There are no comparable data for MLV rabies vaccines, although the best guess suggests that the risk to humans is greater than that from vaccinia.

Bait Development

A successful bait must attract the target species from some distance, and stimulate the individual to eat it. Baits to deliver rabies vaccine need to remain attractive for only 2 or 3 weeks, since the vaccine will be ineffective after that. Foxes have been the major targets to date. They are attracted to a wide variety of meats, fats and cheeses, with no substance having clearly superior attractiveness (82). In the end, ease of mass production and lower cost have been the dominant considerations for choosing attractants.

Placement of vaccine in the bait affected the design of the bait. Early attempts to mix vaccine directly with the bait resulted in

destruction of vaccine activity (57). In Ontario, we experimented with a wax-covered sponge to contain the vaccine (58,83), but by 1987 we used a plastic blister-pack within the bait, similar to that used in Switzerland and Germany (76,84). The blister-pack was embedded in a matrix of beef tallow and wax, containing coloring, scent attractants and tetracycline (as a biological marker: see below) (85).

Experiments to determine best characteristics of baits for raccoons and skunks are just beginning. Debbie (57) reviewed earlier attempts. There is active research in progress to find better baits for raccoons, to combat the recent outbreak of rabies in the mid-Atlantic states (86, and D.H. Johnston, personal communication).

The Ontario Rabies Research Program

The basic structure of the Ontario rabies research program is shown in Fig. 3. We wished to determine critical parameters for rabies control before trying to apply vaccine-baits over large areas. A comprehensive simulation model of rabies outbreaks, including provision for vaccination at various rates, is at the heart of the planning process (31). The process of assembling and testing the model has provided much insight into the reasons for persistence of rabies in southern Ontario (described by Tinline in this volume). The model is currently being used to estimate the proportions of a fox population which must be immunized in order to reduce or eliminate rabies. Those simulations included a variety of starting conditions relating to landscape characteristics and fox population parameters. Such exercises have proved particularly useful because the current performance of the vaccine-bait distribution system is marginal for effective control.

Distribution of baits from low-flying aircraft has been a major thrust of the Ontario program. Early in the history of experimental baiting we determined that placement from the ground was too labor-intensive to be affordable (82,87). Three staff plus a pilot can distribute baits over 500-600 km^2 in a single day, using a Cessna 172. In fact, only 2 staff are in the aircraft each flight, but airsickness is sufficiently frequent that the availability of a standby crew seems essential.

European workers have placed baits by hand (76,84), usually by employing volunteers. Ground placement, if there are people with

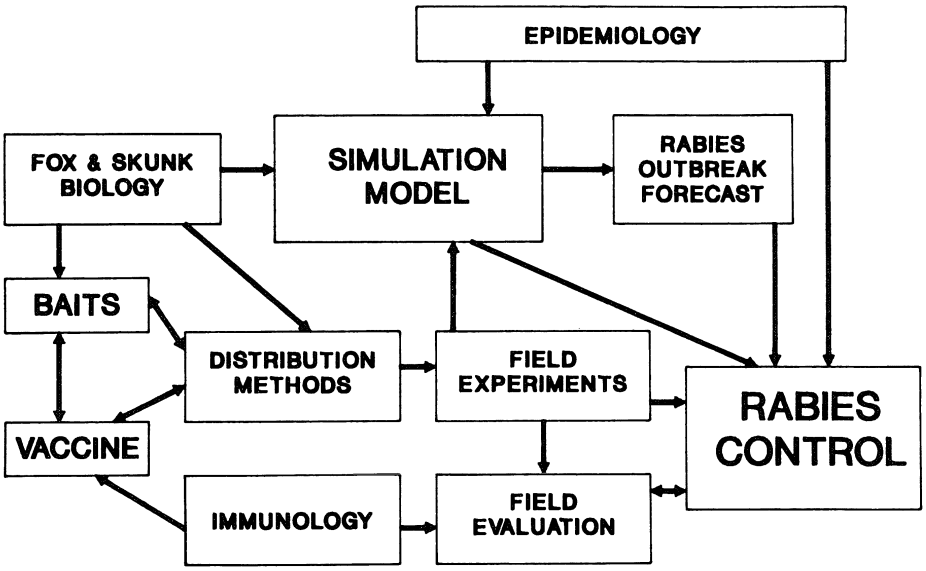

Figure 3. The organization of subject investigations which make up the Ontario Rabies Research Program.

intimate knowledge of the local landscape, offers the chance to place baits where they are more likely to attract foxes. It certainly allows baits to be placed where they will be less conspicuous to crows and humans. Crows removed over 50% of air-dropped baits within 5 days in some Ontario experiments (85). On the basis of estimates done around 1980-82, ground placement in Switzerland cost 1½ times as much as aerial dropping in Ontario, exclusive of labor (82).

Many hunters and trappers' groups have volunteered to help distribute baits in Ontario. We do not believe that we can use these to achieve the universal coverage required to control rabies, but such groups have considerable potential to supplement air-dropping, especially in built-up areas.

Experiments with ground meat baits in Ontario showed no increase in acceptance of baits by foxes over a range from 18-48 baits/km^2 (Table 2), although more skunks took baits at the higher densities (82). Sponge, blister-pack and ground beef baits dropped at 18-23/km^2 all reached 60-65% of foxes on any given area (82,85).

All experimental bait drops have been conducted during late September or early October. These times were chosen to fit the season during which trappers took the largest numbers of foxes, since evaluation of success depended upon specimens obtained from local fur trappers (see below). Over 80% of animals were taken between 15 October and 15 November.

Rules for dropping baits from aircraft were formulated with several considerations in mind. Southern Ontario was originally surveyed by the British army; as a consequence the roads along which farms were established are usually parallel to each other and uniformly spaced at 1.7-2.4 km intervals. As a result, there is often an irregular strip of woodland midway between the roads. Frequently, there is

Table 2. Acceptance of Air-dropped Baits by 3 Wild Species, Huron County, Ontario.

Bait type	Year	Area (km^2)	Baits/ km^2	Percent acceptance[a]					
				Fox		Skunk		Raccoon	
				<28	T	<28	T	<28	T
30 g ball[b,e] of ground meat	1977	549	18	48	52	25	32	-[c]	9
	1976	508	23	74	72	25	34	7	8
	1976	508	24	70	70	38	35	2	9
	1980	549	31	67	-	44	-	30	-
	1976	503	48	63	43	54	56	22	9
Wax-covered[d] sponge	1984	542	18	64	53	34	38	24	29
	1985	542	20	63	64	30	33	44	43
	1986	760	21	61	55	26	25	7	5
Wax-covered blister-pack	1987[e]	340	21	67	69	-	24	-	47
	1987[f]	290	23	50	66	-	23	-	24

[a] An animal was deemed to have eaten a bait if there was a tetracycline deposit in a canine tooth (82,85). <28: % acceptance of baits within 28 days of dropping; T: % of total number accepted.

[b] In 1976 and 1977, baits were made from deadstock meat, including intestines. In 1980, Ontario grade A ground beef was used.

[c] No sample collected.

[d] Liver slurry was added as attractant in 1984-86; a 15 g ball of grade A ground beef was added to each bag in 1986.

[e] Baits dropped in 17 x 23 x 0.0025 cm clear plastic bags.

[f] Baits dropped without bag.

also woodland along the banks of larger streams and rivers. Radio-telemetry studies showed that fox activity was greater along the edges of the woodland than closer to the roads. Consequently, 2 flight lines were used between each pair of roads, one on each side of the woodland strip (82). After several experiments, we determined that baits should be dropped in all habitats overflown, except for areas omitted for safety reasons.

Radio-telemetry observations and bait trials indicated that flight lines close to the roads would increase acceptance by skunks. Hand placement of baits around buildings should also be effective, but because current MLV vaccines do not immunize skunks orally, specific experiments have not been conducted.

Baits were not dropped close to areas of human activity, because of concerns with safety of the vaccine. We sought to minimize human contact with baits, both by dropping away from farms, towns and recreation areas, and by placing great emphasis on communications. Every school in any test area was subject to a presentation by a public health nurse, giving the message to children to leave the baits alone. Local newspapers, radio and television stations were all contacted to publicise the program. Each bait carried a label which included a toll-free telephone number if information was desired. We had agreed with local health authorities that anyone exposed to vaccine should receive post-exposure rabies treatment, but that has not been necessary after 3 years of trials with vaccine.

Evaluation of Field Trial Success

The principal means of estimating the success of different baits was through examination of carcasses of foxes, skunks, raccoons and coyotes obtained by local fur trappers. Each bait contained tetracycline (100-150 mg, ref. 82). Trappers were paid $1 for each carcass, after they had removed the pelt, provided that they supplied location, date of death and other information. When vaccine was included in the experiments, the trappers were trained to take blood samples, and were paid $5 per sample ($7 for skunk blood). They supplied good samples from over 90% of animals taken.

Presence of tetracycline in teeth was the sole criterion for measuring bait acceptance. Thin sections, cut directly from the tooth

(59), were examined for fluorescence under ultraviolet illumination. Examination of teeth taken in years when no baits were distributed indicated no more than 3% of animals showing tetracycline deposits (presumably obtained by scavenging dead livestock which had been treated with that antibiotic). No adjustments were therefore made to trial data to compensate for ambient tetracycline levels.

Acceptance values are listed in Table 2. The stability of fox acceptance over a variety of baits and experimental conditions is striking. Experiments were conducted on areas ranging from 290-760 km^2, which caused considerable difficulty in estimating the true acceptance rate since experiments were all conducted early in the period when young foxes were dispersing most actively (88,89). Dispersal led to ingress and egress to and from the baited areas in the weeks after the baits were placed. In Ontario, juvenile male foxes settled an average of 26-27 km from their natal den, while females moved about 7 km (31; see also Tinline, this volume). The result is that some which were in the experimental area when baits were dropped must have moved out before trapping began, and others must have moved into the area after baits were depleted in the field, but before trapping began. Therefore, acceptance estimates for foxes trapped within 28 days after the baits were dropped are tabulated separately. In bait trials undertaken before vaccine was incorporated, air-drops were made just before trapping began in mid-October. From 1984 on, the drop was made about 20 September, to allow foxes to seroconvert before trapping began. In those years the trapped sample was split between the pre- and post-28 day periods, and the overall acceptance was lower than the estimate for foxes taken in the first 4 weeks after the drops. This indicated that acceptance values obtained for foxes from the Ontario trials are probably lower than would be obtained if the trials had covered very large areas (82,85). Dispersal usually did not cause a comparable problem in skunks or raccoons (Table 2), an observation consistent with radio-tracking studies of those 2 species (D. Voigt, personal communication).

Tetracycline marks gave another very important kind of information. In juvenile foxes the teeth are still growing rapidly enough in October and November that daily growth lines are evident (93). Given

Table 3. Antibody Prevalence after Field Trials of Baits Containing MLV Rabies Vaccine, Huron County, Ontario.

Year	Vaccine[a]	Tetracycline markings				
		Positive			Negative	
		% Antibody-positive		N[b]	% Antibody-positive	N
		Total	Corrected			
1985	ERA^R	6	N.D.[c]	86	0	45
1986	ERA/BHK-21	34	45	121	0.5	401
1987	ERA/BHK-21	36	N.C.[d]	45	10	20

Animals were counted as being serum-positive if they had a FIMT titer >=1/16 *and* an ELISA titer of >0.12 absorbance units (92; see also Campbell and Barton, this volume). All animals were trapped within the test area.

[a] ERA^R = Convac-ERA^R (Connaught Laboratories, Ltd., Willowdale, Ontario); ERA/BHK-21 = ERA virus grown in BHK-21 cells (see Crick and King, this volume).

[b] Total number of samples.

[c] Not done.

[d] Not completed.

the date the trapper killed a fox, we could determine the exact days on which the animal had eaten baits. This allowed us to estimate the proportions of animals which picked up baits in various intervals after the baits were dropped, to compare with the expected duration of effectiveness of the vaccine. It also allowed calculation of the time required for seroconversion in the field.

The first time a MLV rabies vaccine was used in the field in North America was the 1985 experiment. Table 3 shows the rates of seroconversion in foxes; skunks and raccoons are not shown because antibody prevalence was the same in both experimental and control areas. In 1985 the number of seropositive foxes was only slightly (and not significantly) higher than background levels. In a control area, more than 60 km from the test area, 4/141 (2.8%) of foxes had significant antibody, and none were tetracycline-positive. Laboratory trials had indicated that the baits should vaccinate 50% of foxes (58), and the reasons for the failure to vaccinate any foxes are unclear.

The 1986 trial was moderately successful, and preliminary results indicate a somewhat higher rate of seroconversion in the 1987 trial. The corrected seroconversion rate was calculated by removing from the sample all foxes which had eaten their first bait less than 16 days before they were killed. Examination of the daily growth lines in teeth of juvenile foxes showed that none seroconverted in less than 16 days.

The overall vaccination rate in the 1986 trial was 27%, the product of a 61% bait acceptance (Table 2) and a 45% seroconversion (Table 3).

Parameters for Control of Rabies

What proportion of a wild vector population must be vaccinated in order to control the spread of rabies? There clearly is strong interaction between landscape, fox densities and the spread of rabies (29, 88,90,91). The actual fox density at which rabies will die out is still a matter for debate (25,31,94). Therefore, the proportion of a vector population which must be vaccinated in order to control rabies is unknown, but will almost certainly vary from place to place, and from time to time in each locality. The Ontario simulation model (31) indicates that if 60% of a fox population (living under the conditions of southern Ontario) were vaccinated, rabies would die out in over 70% of simulations. When 70% of the population was vaccinated, rabies died out almost invariably. That 70% figure is widely recognized as the threshold for control of urban dog rabies (95). However, the model is quite sensitive to the timing of vaccination with respect to the rise and fall of rabies and fox density (R.R. Tinline, unpublished). Also, unless the "study area" within the model exceeds about 4,000 km^2, rabies would not persist even in the absence of vaccination. There were also indications that a modest increase in trapping, combined with vaccination, applied at the right time in the fox/rabies 'cycle', might increase the probability that rabies would die out. Clearly there is a great deal more to be learned from the simulation models, but model predictions must also be tested in the field, with appropriate verification.

Control of rabies in urban areas may be effected by live-trapping, vaccinating by injection, and releasing, skunks or raccoons (2). At present this is a stop-gap measure, to provide a proactive program

while awaiting development of a vaccine-bait combination suitable for these 2 species. However, the logistics and safety considerations of baiting in cities may indicate that Trap-Vaccinate-Release is a more effective method, although it costs more.

In 1988 figures, it costs about CDN$15-20/km^2 to air-drop baits containing MLV rabies vaccine at 20 baits/km^2. This figure includes the price of the baits, as well as logistic and staff costs: the cost of ground baiting in France, using hired staff, has been estimated at twice that (M. Artois, personal communication). Cost of the urban trapping program is probably around CDN$650/km^2 (R. Rosatte, personal communication).

DISCUSSION

There is cautious optimism that red fox rabies can be eliminated by large scale vaccination. Certainly the success of the Swiss and West German campaigns (84; Wandeler, this volume) is encouraging, although it is too early to say that rabies will be totally eliminated. If the V-RG vaccine is approved for field use, raccoon rabies also appears to be vulnerable.

Elimination of skunk rabies is less certain at the present. The persistence of rabies in skunks in the Mississippi Valley is puzzling. There have been quite long intervals without reported cases in many localities, and the total number of cases was much lower per unit area than in the raccoon or (Ontario) fox outbreak areas. Current theory suggests that at low densities, rabies should be easier to eliminate, yet the Alberta experience contradicts that. Skunks will take bait, and so with a suitable vaccine local outbreaks may certainly be controlled. However, the area of enzootic skunks rabies in the middle of the North American continent is so large, diverse and diffuse that it is harder to predict whether the disease can be eliminated completely. Vampire bat rabies is the largest single disease problem of domestic animals in the American tropics, estimated to cause economic losses exceeding US$50 million per year (1). The control methods are both effective and low-cost, yet wide-scale application was slow to start. Since 1982, over 100,000 cattle have been injected annually with warfarin (2,3), and an additional 3 million cattle are vaccinated annually against rabies.

Large-scale attempts to immunize foxes against rabies should begin in Ontario in 1988. Control of raccoon rabies in the U.S.A. by aerial baiting should follow. Nevertheless, humans will still be at risk to rabies from bats, and probably from skunks, for some time to come. Given the rapid progress towards control of rabies in terrestrial wildlife during the 1980s, however, a dramatic reduction of rabies in terrestrial wildlife seems imminent.

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18

CONTROL OF URBAN RABIES

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ABSTRACT

Justification for the establishment of urban rabies control programs and the description of those implemented in Greater Buenos Aires, Argentina, Lima and Callao, Peru, and Sao Paulo, Brazil, are discussed. At the time of initiating those programs, rabies cases in dogs amounted to over 4,000/year in Greater Buenos Aires (1976), more than 1,000 in Lima and Callao (1982) and about 900 in Sao Paulo (1969); they were reduced to zero in 9, 4 and 16 years respectively. Recommendations for programs in developing countries are presented.

INTRODUCTION

Rabies is a dreadful disease which has concerned health authorities for centuries. Human cases reported in the Americas during the period 1976-1985 are presented in Table 1. Most of them occurred in urban areas (1). Total cases during that period averaged about 300 annually.

Although several excellent vaccines have been developed to prevent the disease in humans, they do not deal with the source of the problem (2). As for any other zoonosis (3), the most cost-effective approach for the control of rabies is through the animal reservoir (4) rather than man. Worldwide, approximately 90% of the human cases are caused by dogs (1,5), even in Europe where canine rabies accounts for only 7.4% of total animal cases (6). Also, most human post-exposure treatments against rabies in the U.S.A. are due to dog bites (7), in spite of the few cases reported in this species.

Because of those reasons, dogs have become the target for preventing the disease in human beings. Scandinavian countries eradicated rabies long before Pasteur developed the first vaccine by eliminating stray dogs and enforcing sanitary policy measures (8). Prussia in 1875 and Singapore in 1892 (9), when their populations were still low, also succeeded in eliminating rabies with the same procedures. Programs based on the leashing and muzzling of dogs have always been unpopular and it was not until simpler vaccines, such as the Umeno-Doi, Semple, chicken embryo (CE) or suckling mouse brain (SMB) (see Bunn, this volume) became available, that programs succeeded in controlling rabies (10) in moderately populated areas such as Memphis and Houston counties, U.S.A. (8), Malaysia (11,12), Tokyo (13), and Santiago, Chile

Table 1. Cases of Rabies in Humans, The Americas, 1976-1985[a]

Countries	Years										Total
	1976	1977	1978	1979	1980	1981	1982	1983	1984	1985	
Argentina	18	8	9	3	0	3	1	1	3	0	46
Bolivia	1	2	10	6	11	6	13	17	3*	7	76*
Brazil	99	140	139	148	170	139	125	99	78*	52	1,189*
Canada	0	1	0	0	0	0	0	0	1	1	3
Colombia	1	5	10	7	25	27	18	-§	11	13	117*
Cuba	1	0	0	0	0	0	0	0	0	0	1
Dominican Republic	3	4	1	0	4	2	1	5*	10	4	34*
Ecuador	13	23	19	25	18	33	44	34*	15*	16	240
El Salvador	12	12	10	11	11	10	12	21	33	24	156
Guatemala	1	4	5	10	5	3	10	5	4	7	54
Haiti	4	1	6	0	2	8	1	0	1*	2	25*
Honduras	14	10	0	0	5	10	11	3	6	5*	64*
Mexico	61	34	84	59	40	59	45	47	63	84*	576*
Nicaragua	1	3	3	1	5	2	5	1*	0	0	21*
Paraguay	2	1	1	1	6	5	2	1	2	3	24
Peru	21	9	12	17	6	29	34	21	32	22	203
U.S.A.[b]	2	1	4	4	0	2	0	3	3	1	20
Venezuela	5	6	9	11	6	16	10	3*	6	4	76*
Total	259	264	322	303	314	354	332	261*	271*	245*	2,925*

[a] PAHO/WHO/CEPANZO Rabies Surveillance for the Americas.

[b] US/PHS/CDC Rabies Surveillance.

* Incomplete data.

§ Data not available.

(14), in which regions between 50,000 and 250,000 dogs were vaccinated and sanitary policy measures applied. At the beginning of the 1970s, the American countries established the goal of controlling canine rabies by the end of that decade (15). Table 2 shows how far they were from reaching that goal by the end of 1979. In 1983 the same countries set for themselves a rather more modest goal: to eliminate rabies from large cities by 1990 (16). The reduction of dog cases during the last 3 years (Table 2) indicates that some of the countries, particularly

Table 2. Cases of Rabies in Dogs, The Americas, 1976-1985[a]

Countries	Years										Total
	1976	1977	1978	1979	1980	1981	1982	1983	1984	1985	
Argentina	4,811	2,334	1,115	1,033	550	469	160	95	95	55	17,717
Belize	10	2	0	1	7	3	0	2	0	0	25
Bolivia	86	280	501	672	718	1,184	593	1,045*	776*	1704*	7,559*
Brazil	4,735	5,231	1,414	4,510	4,551	2,850	2,411	2,250	1,057	389	29,398
Canada	87	73	79	91	51	83	116	63	58	67	768
Chile	4	11	4	2	0	6	0	0	0	0	27
Colombia	1,577	2,117	3,051	2,210	2,043	5,939	1,716	-§	994	717	20,364*
Costa Rica	25	11	0	0	2	0	0	0	0	0	38
Cuba	45	45	43	43	26	35	26	16	26*	15	320*
Dominican Republic	140	86	55	50	82	194	99	138*	132	228	1,204*
Ecuador	198	927	345	1,342	2,273	1,515	1,903	1,427	863*	516	11,309*
El Salvador	57	68	39	55	63	119	40	64	43*	-	548*
Grenada	5	2	5	0	-	5	1	8	9*	1*	36*
Guatemala	79	201	472	310	273	354	331	227	194	346	2,787
Haiti	8	2	2	-	64	61	72	92	40*	55	396*
Honduras	72	129	156	225	1,410	227	228	151	156	117*	2,871
Mexico	3,937	11,705	14,109	11,978	9,903	2,025	2,127	2,455	3,633	1,424*	63,296*
Nicaragua	40	68	270	169	268	42	79	67	62	118	1,183
Paraguay	208	206	284	183	291	187	243	267	201	207	2,277
Peru	407	595	871	792	362	1,452	1,913	2,566	840	505	10,303
Trinidad-Tobago	1	0	0	0	0	0	1	0	0	0	2
U.S.A.[b]	116	120	119	196	247	216	153	132	97	185	1,585
Venezuela	325	502	698	558	389	2,910	455	343	163	318	6,661
Total	16,973	24,715	23,632	24,420*	23,573*	19,876	12,667	11,408*	9,439*	6,967*	173,670*

[a] PAHO/WHO/CEPANZO Rabies Surveillance for the Americas.

[b] US/PHS/CDC Rabies Surveillance.

* Incomplete data.

§ Data not available.

Argentina, Brazil, Colombia, Ecuador, Mexico and Peru, are seemingly succeeding in attaining that goal.

We will discuss the elimination of rabies in three large urban areas of Latin America: Greater Buenos Aires (GBA), Argentina (17,18), Lima and Callao (LC), Peru (19) and Sao Paulo (SP), Brazil (20), all of them far more densely populated than those of the afore-mentioned programs (8,11-14).

PROGRAMS

The 3 programs were implemented in metropolitan areas of developing countries, where most cases of rabies are reported (21). Health education, canine vaccination and stray dog control activities were similar in the three programs, although some differences were observed.

Table 3. Annual Number of Rabies Cases and Reduction Percentages[a] of Animal Rabies in Three Latin American Control Programs, 1967-1986

Year	GBA		LC		SP	
	Cases	%	Cases	%	Cases	%
1967	737		286		1,212	
1968	613		49		1,177	
1969	364		144		989	0.0
1970	263		541		591	40.2
1971	344		208		643	35.0
1972	229		7		573	42.1
1973	872		8		553	44.1
1974	834		8		299	69.8
1975	1,261		4		200	79.8
1976	4,108	0.0	0		195	80.3
1977	1,528	67.9	0		292	70.5
1978	692	83.1	1		209	78.9
1979	539	86.8	0		146	85.2
1980	214	94.7	43		86	91.3
1981	136	96.7	532		76	92.3
1982	31	99.2	1,023	0.0	16	98.4
1983	15	99.6	172	83.2	4	99.6
1984	1	99.9	53	94.8	1[b]	99.9
1985	0	100.0	16	98.4	0	100.0
1986	2[c]	99.9	0	100.0	1[d]	99.9

[a] Since the program was started.

[b] Case from out-of-town.

[c] Non-hematophagous bats.

[d] Goat.

Description of the Areas

GBA is composed of the 19 counties that surround the city of Buenos Aires, the country's Federal District. Although Buenos Aires and GBA form a single metropolis and are an epidemiological unit, the program for Buenos Aires will not be discussed here because its affluence made easier the control activities of the authorities responsible for the program in the city.

Of the more than 10 million inhabitants of the metropolitan area of Buenos Aires, 7 million live in GBA, where rabies was enzootic (Table 3) and its epidemiological cycles depended on the control activities undertaken in the area. The dog population was estimated by periodic samplings in Avellaneda and some other of the 19 counties, to be between 1 dog per 4 inhabitants in 1967 and 1:6 in 1983 (unpublished data). The "Dirección de Control de Zoonosis Urbanas" (Urban Zoonoses Control Division) dependent on the Ministry of Health of the Province of Buenos Aires, coordinated the program with the health authorities of the 19 counties.

The health workers of LC (approximately 6.5 million inhabitants) had successfully controlled rabies during the first half of the 1970s, but in 1980 the disease was reintroduced from neighboring areas (Table 3). Dog population was estimated at 10% of the human population (22). The "Centro Antirrâbico de Lima" (Lima's Rabies Center), dependent on the Ministry of Health, was responsible for the program and coordinated control activities with the health staff of several districts.

The city of SP and 38 different counties of the State of Sao Paulo constitute a metropolitan area with 14 million inhabitants. The program referred to in this paper comprised only the city itself with currently 10 million people. Rabies was enzootic (Table 3) and, as in LC, the dog population was estimated on the basis of the 1:10 ratio. The "Centro de Controle de Zoonoses" (Center for Zoonoses Control), dependent on the municipal health authorities, took over the program in 1973.

Dog Vaccination

The programs were initiated in 1969 in SP, in 1976 in GBA and in 1982 in LC. The goal set for GBA and for LC was to vaccinate 80% of the estimated dog population in three months; for SP it was to immunize

60% in the period ranging from 1 year at the beginning of the program to 12 days after 1978. When the conditions so advised it, focal immunization was established in the three areas. The institutions in charge of vaccination in GBA had the cooperation of veterinary students. "Mission Bioforce" from France cooperated with the vaccination staff of LC during 1985 and 1986 and the Pan American Health Organization provided technical assistance.

Most of the SMB and suckling rat brain vaccines used for the program in GBA were purchased from local commercial laboratories and their quality tested by the federal government. The PAHO/WHO Pan American Zoonoses Center (CEPANZO) also controlled them during the first half of the program. In LC, vaccines prepared with attenuated or inactivated virus from cell culture origin were used in most cases;

Table 4. Numbers[a] and Percentages of Estimated Canine Population Vaccinated in Three Rabies Latin American Control Programs, 1967-1986

Year	GBA		LC		SP	
	Vaccinees	%	Vaccinees	%	Vaccinees	%
1967	235.9	18.4	88.3	27.9	-§	-
1968	311.2	24.3	9.8	3.0	-	-
1969	422.5	33.0	11.3	3.3	69.8	12.1*
1970	445.2	34.8	104.0	29.5	218.6	36.9
1971	422.5	33.0	139.1	38.0	182.6	29.7
1972	268.9	21.0	263.4	69.4	329.0	51.7
1973	498.1	38.9	107.9	27.5	365.6	55.4
1974	438.2	29.2	65.0	16.0	398.6	58.2
1975	322.8	21.5	156.2	37.1	452.2	63.7
1976	974.1	64.9*	61.3	14.1	529.8	72.0
1977	974.0	64.9	25.1	5.6	525.0	68.9
1978	1016.5	67.7	25.9	5.5	614.3	77.7
1979	1310.3	97.0	15.5	3.2	657.0	80.2
1980	1166.4	86.3	82.1	16.4	700.4	82.5
1981	1268.8	93.9	36.8	7.1	766.6	87.4
1982	887.5	65.7	348.0	64.8*	780.5	86.6
1983	777.8	65.9	41.2	7.4	794.3	85.8
1984	1175.9	99.6	86.6	15.0	791.4	81.4
1985	439.1	37.2	376.1	63.1	743.6	74.9
1986	865.9	73.4	25.2	4.1	656.3	63.4

[a] In thousands.

* Program initiation.

§ Data not available.

these vaccines were either imported or donated by the Maltese Cross Association. In SP, CE vaccine was employed during the first 7 years. After that, SMB vaccine produced in an official laboratory and tested by federal agriculture authorities, was used.

Vaccination was carried out on a door-to-door basis (23) or in vaccination clinics. The number of vaccinated animals and the coverage of the 3 programs are shown in Table 4.

Dog Population Control

The capture of dogs was awarded low priority in the 3 programs. In addition, the training of the staff responsible for this task emphasized health education, to avoid the image of dog killers. The number and percentages of the dog population eliminated every year are presented in Table 5. The percentages ranged between 0.4 and 14.9,

Table 5. Numbers[a] and Percentages of Estimated Animal Population Eliminated During 3 Rabies Control Programs in Latin America, 1967-86

Year	GBA		LC		SP	
	Number	%	Number	%	Number	%
1967	83.6	6.5	134.6	42.5	-§	-
1968	78.8	6.1	96.1	29.3	-	-
1969	103.1	8.0	120.4	35.4	14.6	2.5*
1970	137.5	10.7	41.2	11.2	47.0	7.9
1971	122.4	9.6	114.6	31.3	23.9	3.9
1972	125.0	9.8	58.9	15.5	28.2	4.6
1973	72.0	5.6	62.2	15.8	39.4	10.8
1974	64.4	4.3	49.5	12.2	35.5	8.9
1975	41.9	2.8	54.6	13.0	30.1	4.2
1976	106.3	7.1*	36.1	8.3	36.4	5.0
1977	83.3	5.5	36.2	8.0	69.1	9.1
1978	106.8	7.1	26.3	5.6	67.9	8.6
1979	129.2	9.5	11.8	2.4	65.1	7.9
1980	145.4	10.8	21.9	4.4	69.1	9.1
1981	161.0	11.9	73.6	14.2	63.8	7.3
1982	111.4	8.2	80.2	14.9*	62.5	6.9
1983	35.1	3.0	59.0	10.6	50.8	5.5
1984	17.8	1.5	2.5	0.4	49.6	5.1
1985	25.1	2.1	2.3	0.4	42.6	4.2
1986	17.5	1.5	2.7	0.4	39.6	3.8

[a] In thousands.

* Program initiation.

§ Data not available.

Table 6. Persons[a] Bitten and Undertaking Treatment in 3 Areas of Latin America, 1967-1986

Year	GBA		LC		SP[b]	
	Bitten	Treated	Bitten	Treated	Bitten	Treated
1967	58.3	21.4	11.0	4.7	-§	-
1968	51.9	17.7	10.1	3.5	-	-
1969	79.6	18.8	11.6	4.7	-	-*
1970	87.9	24.3	17.8	8.2	-	-
1971	48.5	20.5	22.0	8.4	-	-
1972	43.8	18.8	16.8	4.6	-	-
1973	67.3	30.6	22.9	5.6	-	-
1974	63.5	28.3	23.0	6.7	-	-
1975	63.7	28.5	23.4	6.9	-	-
1976	93.6	45.5*	24.2	6.3	26.9	12.2
1977	75.9	28.5	19.5	5.1	33.0	14.2
1978	74.5	23.3	17.4	4.4	35.9	15.7
1979	79.3	24.2	14.2	4.1	40.0	16.2
1980	66.7	19.1	11.9	3.8	40.0	17.5
1981	122.3	28.8	13.5	5.6	41.9	18.2
1982	84.1	20.2	24.8	13.2*	42.6	19.5
1983	76.1	16.6	15.4	5.6	43.6	18.1
1984	68.6	17.0	10.1	2.9	55.9	21.1
1985	86.7	15.7	7.9	2.5	47.6	14.9
1986	67.8	13.3	8.9	2.7	43.9	9.0

[a] In thousands.

[b] Source: Comissao Permanente de Controle da Raiva, Sao Paulo.

* Program initiation.

§ Data not available.

i.e., below the figures generally recommended. When comparing Tables 4 and 5, it may be seen that during some of the years more than 100% of the estimated population was vaccinated/eliminated. This indicates that many dogs were vaccinated twice, or that either the population may have been underestimated or some vaccinated dogs eliminated.

Both the number of dogs vaccinated and eliminated in the 3 cities was different. These figures were highest in GBA where dog population was the greatest probably because more food was available. As the program advanced and Argentina's economic situation deteriorated, the ratio of dog: human decreased.

Human Rabies Treatment

In the 3 countries, persons bitten by rabid animals were immunized with the SMB vaccine. The doses administered per treatment were 9-10 in GBA, 14 in LC and, in SP, 35 at the beginning of the program and 10 during the last years of the period reported. Table 6 shows the number of persons bitten by animals suspected of being rabid and of those vaccinated between 1967 and 1986 in the areas of the 3 programs.

Achievements

As far as we know, the elimination of rabies from cities as big as GBA, LC and SP has no precedent in the history of the disease. Rabies was eradicated from currently densely populated urban areas when their populations were smaller.

At the beginning of the programs, animal cases in GBA amounted to 85% of the country's total, in LC to 53% and in SP to 34% (Tables 2 and 3). Cases in animals in GBA and LC declined to zero in 9 and 4 years

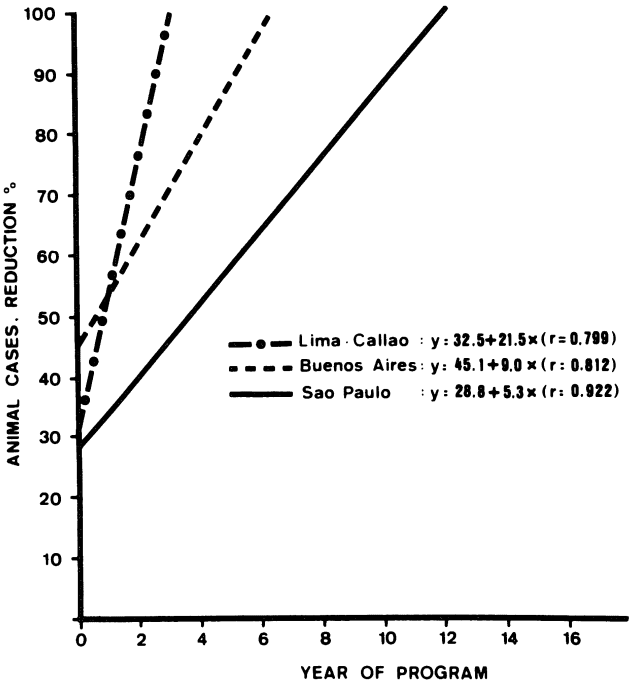

Figure 1. Linear regressions of animal rabies case reduction in 3 areas of Latin America.

Table 7. Number of Human Rabies Cases and Rate/100,000 in 3 Areas of Latin America, 1967-1986

Year	GBA		LC		SP	
	Cases	Rate	Cases	Rate	Cases	Rate
1967	9	0.16	3	0.09	20	0.37
1968	10	0.19	1	0.03	18	0.32
1969	5	0.09	1	0.03	5	0.09*
1970	4	0.07	3	0.08	8	0.14
1971	2	0.04	2	0.05	8	0.13
1972	0	0.00	0	0.00	12	0.19
1973	7	0.13	0	0.00	11	0.17
1974	6	0.10	1	0.02	5	0.07
1975	4	0.06	0	0.00	4	0.06
1976	10	0.16*	0	0.00	0	0.00
1977	4	0.06	0	0.00	4	0.05
1978	8	0.13	0	0.00	3	0.04
1979	2	0.03	0	0.00	2	0.02
1980	0	0.00	2	0.04	0	0.00
1981	1	0.01	4	0.07	3	0.03
1982	0	0.00	14	0.25*	0	0.00
1983	0	0.00	10	0.17	0	0.00
1984	0	0.00	1	0.02	0	0.00
1985	0	0.00	1	0.02	0	0.00
1986	0	0.00	0	0.00	0	0.00

* Program initiation.

respectively (Table 3). The reduction rate was somewhat lower in SP. The difference in the regression coefficient of the case reduction in LC, when compared with those of GBA and SP (Fig. 1) was statistically significant ($p < 0.05$). Many factors may have accounted for this difference: the dog population was smaller in LC, rabies was epizootic while enzootic in GBA and SP, and a different type of vaccine was employed than those used in GBA and in SP.

Cell culture vaccines used in LC, whether prepared with attenuated or inactivated virus, afford a longer duration-of-immunity in dogs than the ones used in GBA and SP (24-26). Although the latter can be employed successfully in control programs as demonstrated in this paper and elsewhere (27), the W.H.O. Expert Committee on Rabies (4) suggests the use of vaccines with higher potency, as in those prepared in cell culture. The use of these vaccines, which afford 3-year duration-of-immunity, increases the number of immune animals at any single time (28).

As a result of the control of rabies in dogs, human cases were eradicated in GBA and SP. In these two areas the last cases were reported in 1981 and in LC in 1985 (Table 7).

The number of persons who received rabies treatment also decreased as a result of the program (Table 6). Fig. 2 shows the linear regression of the number of persons vaccinated annually in GBA during the periods 1968-1976 ($Y = 15.3 + 2.6X$; $r = 0.822$) and 1976-1986 ($Y = 34.1 - 2.2X$; $r = -0.817$), as well as the projection of the first line for 1976-1986.

As in the case of brucellosis (29), savings resulting from the program can be estimated on the basis of the projected and actual number of persons vaccinated during its implementation (Table 8). Considering that each person undergoing treatment loses at least half a day's work per inoculation, the reduction in the lost working days between 1977 and 1986 would represent a saving of more than US$6 million including a 7% annual adjustment of the actual estimated saving

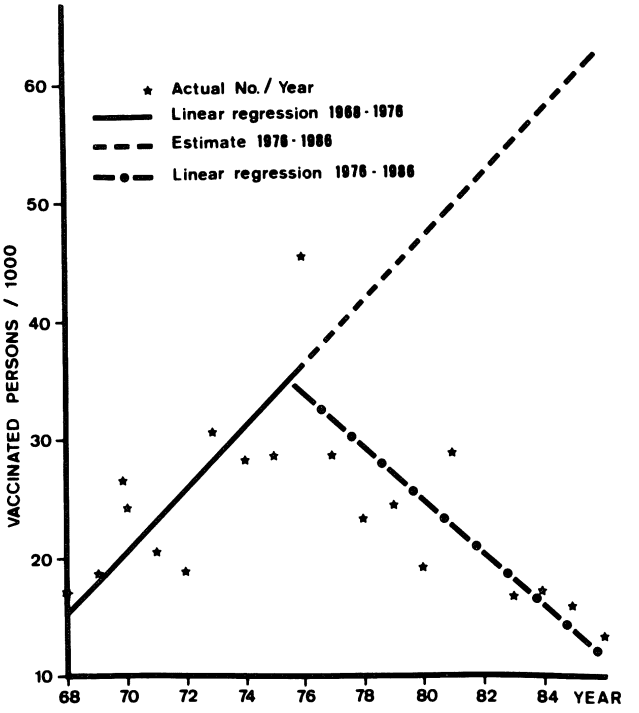

Figure 2. Persons vaccinated against rabies, Greater Buenos Aires, 1968-1986.

(Table 8). Other authors have estimated the cost of vaccination lost labor at approximately $200 (30) or more (28) per person. If we use this figure, the savings would increase more than 10-fold.

RECOMMENDATIONS

Whether enzootic or epizootic, urban rabies can be controlled and even eradicated in a reasonable period of time, provided a comprehensive control program is implemented.

To assure adequate funding for the successful development of a program, the decision to control rabies must be made at the highest levels: the politicians and administrators concerned should be made aware of the financial benefits derived from such a program (Table 8).

Legal support for the program should be obtained. The knowledge of dog ownership pattern, animal: human ratios, canine population density, age structure and turnover, etc., would be helpful in obtaining the approval of legislators. The W.H.O. Expert Committee on Rabies

Table 8. Savings Resulting from the Rabies Control Program, Estimated on the Basis of Persons Treated, Greater Buenos Aires, 1977-86

Year	Number of Persons*		Savings in $	
	a) Estimated	b) Difference	c) Estimated	d) Adjusted
1977	39.1	10.6	176,667	324,794
1978	41.7	18.4	306,667	526,910
1979	44.3	20.1	335,000	537,937
1980	47.0	27.9	465,000	659,611
1981	40.6	20.8	346,667	486,218
1982	52.2	32.0	533,333	699,091
1983	54.9	38.3	638,333	781,786
1984	57.5	40.5	675,000	772,808
1985	60.1	44.4	740,000	791,800
1986	62.3	49.0	816,667	816,667
Total		302.0	5,032,834	6,397,821

* In thousands.

a) Projection of linear regression from Fig. 1.

b) column (a) minus second column of Table 6.

c) Difference (b) x 5 (labor days lost per treatment) x $100 (minimum monthly wage in Argentina)/ 30.

d) 7% per year.

recommends that such data be collected before any program is initiated (4). However, in serious epizootic conditions, the lack of information should not preclude the immediate initiation of control activities.

A central administrative level should be entrusted with planning and coordinating the program activities, while operational aspects should be the responsibility of local level. Both levels should be headed by professionals with experience in preventive medicine and capable of coordinating intra- and extra-sectoral cooperation. Coordination should include official and private institutions, such as professional associations, community groups, schools, universities, societies for animal protection, etc.

Resources should be concentrated in areas of high epizootiologic importance. However, the capacity to react speedily in the event of outbreaks in neighboring free areas should be maintained.

Health education, animal vaccination and epidemiological surveillance should be the program's main activities, while dog population control should be complementary.

Institutions and the community should participate in health education activities, in which the idiosyncrasy of the population should be taken into account and responsible dog ownership stimulated. The dramatic aspects of the disease should be avoided in these activities while emphasizing the importance of adequate care and vaccination of pets.

Dog vaccination should be performed annually, in periods as short as possible, and with a coverage of 70-80% of the estimated dog population. Private veterinarians should be invited to cooperate with the program by vaccinating dogs in the areas where their clinics are located. In some areas better coverage was obtained with door-to-door vaccination than with vaccination clinics; a combination of both methods would render better results.

Obtaining enough animal rabies vaccine has been a serious impediment in several programs. As shown in this paper, excellent results can be obtained with vaccine produced either at official or commercial laboratories, provided it complies with the quality control tests (31). It is not necessary for health authorities to produce rabies vaccine, in the same way that they do not produce all the vaccines

used in other immunization programs; however, they must not delegate the quality control of vaccines.

Human resources for the programs should not be limited to government officials; veterinary students and staff of international, national and community institutions can be invited to cooperate with programs. Training and supervision by veterinarians and other health officials will assure the efficiency of non-professional staff. Training should not be limited to vaccination procedures but should also include cold chain principles, asepsis, good relations with the community, health education, etc.

The identification of rabid dogs and their human and animal contacts, as well as the remittance of samples to diagnostic laboratories should be included in epidemiological surveillance activities. It should be kept in mind that the clinical observation of dogs suspected of being rabid by experienced veterinarians can be as efficient as some of the laboratory methods for rabies diagnosis (32), e.g., Sellers' stain. The fact that the capacity of making accurate clinical diagnosis may be lost when rabies has been controlled in a given area should also be taken into account. Checking the quality of vaccines during the implementation of the program should be also included as a surveillance activity because the loss of vaccine potency before its expiration date has been detected and explained rabies in dogs vaccinated during the program (33).

Appropriate ways of controlling, supervising, monitoring and evaluating activities should be established to determine the extent to which the objectives and goals of the program are being attained, if the standards for quality, production, yield, efficiency and coverage are being complied with and what changes are required to adjust or improve the program.

Local programs should be coordinated with, and whenever possible included in national programs. When rabies has been controlled and the danger of reintroduction from neighboring areas persists, vaccination should either be continued at the same rhythm (4) or spaced further in time (8).

Lastly, health workers should be alerted on the possibility of the emergence of rabies in wildlife after urban rabies has been controlled,

as has happened in other countries and would seem to be happening in the programs reported here (Table 3).

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INDEX

Errata. The following text was inadvertantly omitted from the book. It should be inserted after page 180 and before page 181.

of the extremities who died after 9 days of illness (15). A further isolation of Mokola virus from a shrew was made in 1971 in Cameroon (16).

In 1981, Mokola virus isolations from domestic animals were described by Foggin (17). Over a period of several months, a dog which had been vaccinated previously with a potent inactivated rabies vaccine of tissue culture origin, and 6 cats, all from the same locality of Bulawayo in Zimbabwe, apparently died of rabies. Most had displayed the symptoms of dumb rabies but some showed slight aggressiveness when handled. Neutralization index tests performed in mice confirmed that all 7 isolates were antigenically indistinguishable and different from classical rabies viruses.

In 1984, Saluzzo and coworkers (18) reported the isolation of Mokola virus (An RB3247) from a rodent, *Lophuromys sikapusi*, in the Central African Republic.

Mokola antibodies have been demonstrated in the serum of 1 of 5 *Eidolum helvum* bats shot near Sokoto in northern Nigeria, several other mammalian species, including man, and a great reed warbler (13).

Serotype 4: Duvenhage Virus

In 1971, Meredith and coworkers (19) reported the isolation by mouse inoculation of Duvenhage virus from the brain of a 31 year-old man who died 5 weeks after he had been bitten on the lip, while asleep, by an unidentified bat. He came from the farm Tooyskraal some 100 km northeast of Pretoria, Republic of South Africa. Repeated tests with fluorescent anti-rabies serum on his brain were negative, as were tests on the brains of inoculated mice which died. However, Negri bodies were seen in numerous Purkinje cells of his cerebellum and in the mouse brains.

Eleven years later, a second isolation (SA29) of the virus was made from a bat from Louis Trichardt (12), about 250 km north of the farm Tooyskraal. This bat was also unidentified but was thought to be insectivorous. A third isolate, in 1986, from a *Nycterus thebaica* bat in Zimbabwe (C.M. Foggin, personal communication) completes the list of isolations of Duvenhage in Africa to date.

In the same year (1954) that a serotype 1 virus was first reported in an insectivorous bat in the U.S.A. (20,21), Nicolić and Jeselić (22)