INFECTIVITY AND DISTRIBUTION OF SKUNK RABIES VIRUS IN MICE FOLLOWING ORAL AND PARENTERAL ADMINISTRATION

by

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INTRODUCTION

Rabies is one of the world's oldest and deadliest zoonoses. It is caused by a neurotropic rhabdovirus. Viral proliferation in the central nervous system results in a gruesome clinical disease in man and animals. This zoonoses is present worldwide (Kaplan, 1977). Because of its human and veterinary medical significance, rabies, for centuries, has been the target of extensive studies, especially since the time of Pasteur. Certain aspects of this disease, however, remain unresolved.

One is the question of rabies viremia. The rabies virus is capable of replicating in a variety of non-nervous tissues, including tissue cultures (Wiktor and Clark, 1975). Thus, several workers have considered the possibility of a hematogenous pathway in the spread of the virus in the body of rabid individuals. The missing evidence to support this hypothesis is the failure to regularly demonstrate the rabies virus in the blood.

If rabies viremia occurs consistently, the implications --aside from the pathogenesis--would be great. Oral infection has been demonstrated; hence, viremic blood could be a good common source of rabies infection and transmission, as for example vampire bats (Desmodus rotundus) and arthropods feeding on a bovine host. Noble and Noble (1976) mentioned in their book Parasitology: The Biology of Animal Parasites that "rabies of many mammals in the USSR has been reported as being transmitted by ticks...", and studies on rabies

transmission by ticks continue in that country (Vanag, 1978).

Other implications are related to finding viremia in rabies. It would focus attention on development of much better rabies prophylaxis or therapeutics. The blood could be an appropriate diagnostic specimen, especially in cases where the brain is not available or the disease is not yet terminal. In public health, a generalized distribution of the rabies virus, particularly in the blood, would emphasize the danger of eating raw or inadequately cooked food from rabid animals, handling dead rabies cases as in autopsies, and in receiving tissue or organ transplant from rabid donor. The possibility of these kinds of rabies infection in man are not remote as may be imagined. Rabies in cattle and swine and in wild food-animals occurs in many parts of the world, and the slaughter of animals in the incubation period of the disease could place virus-infected meat and products in the market. In certain areas, particularly the Far East and Africa, local customs of eating raw or undercooked meat could make rabies a real food-related hazard. Lastly, the danger of rabies being acquired from the laboratory or from receiving transplant is already documented in three human cases, the most interesting being that of a woman who got rabies from a corneal transplant (Houff et al., 1979; U.S. Center for Disease Control, 1979).

The present study dealt with rabies viremia in mice infected with a skunk rabies strain by three routes: oral, intramuscular, and intranasal. Parenteral inoculation by the bite is the foremost and classic mode of rabies trans-

mission, but nonbite transmission by inhalation or ingestion of rabies-infected material has been proven and, therefore, had to be considered.

The isolate used was from a naturally-rabid striped skunk (Mephitis mephitis). This wildlife species is one of the leading reservoirs of rabies in the United States, being by far the most commonly reported rabid since 1961 (Parker, 1975).

This investigation also compared the infectivity of the skunk rabies virus by the intramuscular, intranasal, or oral routes. The organ distribution of the virus at the terminal stage consequent to these routes of inoculation was determined.

LITERATURE REVIEW

Rabies history dating back to the records of antiquity and extending up to the middle of the present century has been thoroughly reviewed by Steele (1975). Throughout this period, some ancient mythologies aside, people knew rabies transmission to be exclusively by rabid animal bite or saliva contamination of pre-existing wounds or lesions. Hence, according to Steele, the Roman writer Cardanus described the infectivity of saliva of rabid dogs while Celsus, a first century A.D. physician, emphasized the danger in all rabid animal bites and recommended "caustics, burning, cupping, and also sucking the wounds of those bitten".

In their rabies historical reviews, Tierkel (1971) and

West (1973) pointed out that as early as the 23rd century B.C. rabies was not only a recognized disease in Babylon, but had been written into laws which decreed that "if a dog were mad, and the authorities informed the owner accordingly. and if he failed to keep the animal in and it bit someone, a specified fine became payable..."

Knowledge of the portal of entry for the rabies virus via a bite wound or wound contamination, therefore, dates as far back as the 23rd century B.C., yet, the premise that infection is exclusively by these modes has only recently been amended. During the last two decades rabies has been transmitted experimentally by ingestion (Soave, 1966; Fischman and Ward, 1968; Fischman, 1969; Correa-Giron et al., 1970; Fischman and Schaeffer, 1971; Bell and Moore, 1971; Kovalev et al., 1971; Ramsden and Johnston, 1975; and Charlton and Casey, 1979a and b) and by inhalation (Atanasiu, 1965; Hronovsky and Benda, 1969; Hronovsky, 1971; Fischman and Schaeffer, 1971; and Charlton and Casey, 1979a and b). Research interest in these nonbite modes of rabies transmission arose from observations that they do occur in nature and could have a major role in the epidemiology of rabies.

INTRANASAL ROUTE--AIRBORNE RABIES

Two tragic events in the late 1950's focused attention to nonbite rabies transmission. In 1956 a public health worker died of the disease after entering Frio Cave, near Uvalde, Texas (Irons et al., 1957). Later, in 1959, a mining engineer who had been to the same cave died of rabies (Humphrey et al., 1960). Both victims allegedly had no history of any animal

bite. These deaths stimulated field studies and experiments since they suggested the possibility of transmission by aerosols in the cave, which was known to harbor rabid bats.

An extensive series of field studies, from 1960 to 1966, proved the occurrence of airborne rabies transmission (Constantine, 1967). Constantine (1962) exposed coyotes and foxes in Frio Cave for 24-30 days in cages specially designed for air exposure only; all of the animals became rabid. Sentinel animals held similarly in Frio Cave in the years 1960-1963 and 1964-1966 and those at another cave in 1961 also developed rabies (Constantine, 1967). Subsequently, Winkler (1968) isolated viable rabies virus from the atmosphere of Frio Cave by using a mechanical air-sampler.

Related studies described the extensive use of bat caves by carnivores and provided some statistical analyses that sought to correlate increased carnivore rabies with airborne infection in bat caves (Frederickson and Thomas, 1965; Winkler et al., 1972). Other studies suggested that inhalation of rabies virus could be a mechanism of transmission in terrestial mammals, especially skunks, where there is communal denning with behavioral contact involving a lot of sniffing (Kauker, 1967). Constantine (1967, 1971) believed that terrestial carnivores infected by the airborne route were capable of transmitting rabies to other terrestial mammals.

Not all reported airborne rabies transmission has been directly associated with bat caves. Waterhouse (1970) considered inhalation as a factor in a series of rabies cases

in dogs in a quarantine facility in Great Britain. Winkler et al. (1972) reported an outbreak of rabies in a laboratory animal colony in 1967 as due to airborne dissemination of the virus. Captive foxes, coyotes, and opossums at the U.S. Public Health Service's rabies research station in New Mexico began dying of rabies in an outbreak lasting for 8 months. A total of 64 animals died, and epidemiologic investigations indicated airborne transmission.

Inhalation rabies has also been shown to be a potential hazard for laboratory workers. One worker engaged in the production of rabies vaccines died of rabies from what seemed to be inhalation of aerosols generated during the homogenization of infected goat brains in a blender (Winkler et al., 1973). In another separate incident, a laboratory technician, also working in rabies research, got rabies from inhalation of aerosols during the spraying of modified live rabies virus suspensions in a pharmaceutical manufacturing machine (U.S. Center for Disease Control, 1977).

The relative species susceptibilities to inhalation rabies infection have not been studied. One should assume that all species susceptible to parenteral infection may also succumb following inhalation (Winkler, 1975).

ORAL RABIES TRANSMISSION

Experimental evidence for oral rabies transmission was presented in 1908 first by Fermi and then by Remlinger, as cited by Fischman and Ward (1968). Early rabies authorities, however, considered natural transmission via the oral route to be exceedingly rare, if not impossible. Hutyra et al.

(1949) failed to infect per orally species such as the dog, fox, sheep, and horse, and it was concluded that rabies virus would not survive the gastric juices (Kelser, 1955). Kelser also referred to a case of a woman in an early stage of rabies who apparently transmitted the disease to her nursing baby, and he mentioned a few other instances in which nursing animals became infected possibly through the milk. Teething abrasions in the gum were suspected as the route of infection. Milk from infected animals was shown to contain virus occasionally (Hagan and Bruner, 1961) and consumption of milk from rabid cows was deemed inadvisable, but transmission of rabies in this manner was considered highly unlikely (Hagan and Bruner, 1961; Tierkel, 1963).

Renewed interest in oral rabies transmission was stimulated partly by the findings on the airborne route of rabies transmission, which conclusively showed that nonbite transmission occurs, and partly by the report of Sulkin et al. (1957) on the presence of rabies virus in the brains of baby bats suckling rabid mother bats.

Johnson (1959) surmised that the spotted skunk strain of rabies virus might be spread among skunks by a route other than the bite. His finding of virus in breast tissue led him to speculate that virus might be passed from mother to young via the milk. In a subsequent review, Johnson (1965) stated that mother mice developed rabies following ingestion of their infants dying of the viral infection, but he was uncertain whether the virus had entered via the gastrointestinal tract or the nasopharynx.

Soave (1966), however, stressed that the opportunity for mammalian reservoirs to cannibalize animals dying or rabies may be greater than the potential of inhaling rabies virus. He demonstrated oral transmission of rabies in two groups of mice fed rabies-infected mouse brains, one group with and the other group without a surgical wound in the mouth when fed. Following Soave's work, other studies demonstrated the infectivity of various strains of rabies virus when given via the oral route to a wide variety of animals.

Fischman and Ward (1968) orally transmitted the CVS-fixed strain of rabies virus to neonatal and older mice, hamsters, guinea pigs, and rabbits. They routinely observed oral transmission to mother mice following cannibalism of their previously infected infants. Also, infected mother mice transmitted rabies to their offspring infrequently, but the authors could not tell the exact mode of transmission.

Correa-Giron et al. (1970) successfully infected both suckling and weanling mice. The CVS and three street strains originally isolated from the brain of a bat, cow, and man produced infection in mice following infestion of virus-laden mouse brain tissue. One street strain from the brown fat of a rabid insectivorous bat failed to infect per os.

The susceptibility of various carnivora to rabies virus by the oral route was studied by Bell and Moore (1971) and Ramsden and Johnston (1975). Bell and Moore found that striped skunks (Mephitis mephitis) became rabid from ingesting just one mouse infected with low-passaged isolates from the brain of silver-haired bats (Lasionycteris noctivagans).

Isolates from the hoary bat Lasiurus cinereus and the small brown bat Myotis sp. did not infect ferrets and cats, even when the latter ate up to 25 rabies-infected mouse carcasses (Bell and Moore, 1971). Ramsden and Johnston found that the striped skunk and the red fox Vulpes fulva could be infected fatally, at least with high oral dosages; 10 of 13 young skunks fed 5 to 65 CVS-infected mice, and two of five immature foxes given 5 to 35 mice infected with a skunk rabies strain died of rabies. Ramsden and Johnston stated that results of this study and those of others proved ingestion of rabies-infected carcasses to be a possible means of rabies transmission in carnivores. They cited Kovalev et al. (1971) who recorded rabies deaths in two of three Arctic foxes fed rabid mice and in another fox fed the carcass of a rabid domestic rabbit. Ramsden and Johnston further expressed the view that frozen rabid carcasses in cold climates might provide a source of rabies infection to scavengers for long periods.

A wide variety of rabies street isolates, in addition to the fixed CVS, were used by Charlton and Casey (1979a) in their experimental oral and nasal transmission studies in mice. Street isolates came from the brain of a dog, cat, horse, cow, sheep, red fox, Arctic fox, striped skunk and an Eptesicus fuscus bat, and the salivary glands of skunks. Infected mice brain and the nonpassaged skunk salivary gland isolate were administered orally either by stomach tube, moistened pledget in the mouth, or free choice. Charlton and Casey found that CVS mouse brain suspension and skunk salivary gland

suspension were more infective orally and nasally than the rest of the isolates, but the infectivity was dose-related. Their results indicated that virus of high titer was required to consistently produce infection in mice by these nonparenteral routes.

RABIES VIREMIA

Evidence accummulated since the time of Pasteur overwhelmingly supports the view that the primary mechanism of rabies spread of the rabies virus in the body is via nervous pathways (see reviews by Matsamuto, 1970; Johnson, 1971; Debbie, 1974; Baer, 1975; Schneider, 1975; and Murphy, 1977).

Pasteur and his co-workers (1881) were the first to demonstrate the role of the central nervous system in rabies, and, as quoted by Baer (1975), wrote that "the central nervous system, ...especially the bulb which joins the spinal cord to the brain, are particularly concerned and active in the development of the disease." A few years later, DiVestia and Zagari (1887) showed that the inoculation of rabies virus into peripheral nerves produced rabies and that cutting or cauterizing the sciatic nerve after peripheral inoculation was a life-saving procedure. Since then, many workers have injected rabies virus into nerves to investigate rabies pathogenesis, while many others investigated the distribution of the virus in the body, primarily of the central nervous system, at various periods after peripheral inoculation (Baer, 1975).

The preponderance of work and evidence on the neural pathway of rabies infection and dissemination has left the

question of rabies viremia unsettled. Few investigations of this question exist in the medical literature--some with conflicting results.

Schindler (1961, 1966), Baer et al. (1965), Fischman (1969), and Murphy et al. (1973) all failed to find rabies virus in the blood of experimentally infected animals. Wong and Freund (1951), Reagan et al. (1955), Krause (1957, 1966), Borodina (1959), Frye and Enright (1964), Baratawidjaja et al. (1965), Kitselman and Mital (1967), Shashenko and Kovalev (1971), and Gorshunova et al. (1976) all reported some evidence of viremia in rabies.

Schindler (1961) inoculated 80 mice intramuscularly with 100,000 intracerebral MLD50 of fixed virus. Blood samples collected from 10 mice 25 minutes and daily for one week after inoculation failed to infect mice intracerebrally. He concluded that this absence of rabies virus in the blood stream during the incubation period was evidence that the virus traveled along or in nerves from the periphery to the central nervous system and vice versa.

Baer et al. (1965) studied the pathogenesis of fixed rabies virus in rats. Thirty-two to 100 peripheral LD50 were administered into the rear foot pads. In their viremia study they inoculated the buffy coat of rat heart blood, sampled at 3, 6 and 12 hours, and daily for five days after foot-pad injection, into mice intracerebrally. They got negative results. This, plus the absence of mortality in rats whose sciatic nerve fibers were cut prior to rabies inoculation, convinced these investigators that even a low-

grade viremia did not occur in rabies infection.

Kitselman and Mital (1967) reported viremia in 20 per cent of both neurectomized and non-neurectomized rats, one hour after inoculation of either fixed or street rabies virus into the rear foot pad. This was indicated by the failure of the neurectomies to prevent rabies infection and by the deaths of about 20 per cent of the mice inoculated intracerebrally with one-hour heart blood samples from the infected rats. Kitselman and Mital concluded that the virus bypassed the severed sciatic and saphenous nerves in reaching the brain, proving that a hematogenous pathway existed in combination with or secondary to the neural route.

Murphy and his staff (1973) did a comprehensive study of the pathogenesis of rabies, comparing rabies pathogenesis with the related African viruses, Lagos bat and Mokola. They considered both centripetal and centrifugal spread of the viruses. CVS, vampire bat, and Arctic fox rabies virus strains were used to infect four-day-old Syrian hamsters (Mesocricetus auratus) intranasally, intramuscularly or intrapedally. Histology, immunofluorescence, electron microscopy, and titration by mouse inoculation were done on tissues of infected hamsters killed at 24, 36 to 40, 48, and 60 hours and 3, 4, 5, and 6 days and of surviving animals at 8 to 16 days post-inoculation. They found no evidence of rabies viremia, although the tissue titration was "too insensitive" for detecting the early course of infection and one blood specimen of a Mokola-infected hamster was positive.

More controversial is the question of rabies viremia

in the terminal stage of the disease and whether or not it has a role in the centrifugal spread of the virus from the brain. Schneider (1975) cited several authors who isolated rabies virus from non-nervous organs and, occasionally, from the blood after infection. Most of them believed that the internal organs became infected through the blood during a viremic stage which escaped recognition due to the low sensitivity of the available isolation methods.

Schneider (1975) pointed out that the results of his study (Schneider, 1969), in which mice were injected intravenously with rabies virus, and those of Fischman and Schaeffer (1971), in which oral, nasal, and intravenous routes were considered, did not support the viremic hypothesis. These separate studies showed no evidence of infection of non-nervous organs before brain involvement, nor were peripheral organs terminally infected at a higher rate than the brain.

Evidence for a hematogenous centrifugal spread of the rabies virus was presented as early as 1829 by Hertwich (Burne's letter, 1970). He inoculated blood, saliva, and nervous tissues internally and into fresh wounds of 59 dogs. Fourteen dogs developed rabies from the inoculation of either blood or saliva. Hertwich concluded that these "vehicles" were infective at "every period of the confirmed disease" and even for about 24 hours after the death of the animal.

Russian authors Shashenko and Kovalev (1971) reported a viremic phase in several animal species experimentally infected with a street rabies strain. They detected viremia in sheep, cattle, dogs, foxes, and cats, but only during the

febrile phase prior to the onset of clinical signs.

Intracerebral inoculation, which roughly approximated the stage of rabies centrifugal spread from the brain (since the virus was directly deposited in this organ), suggested occurrence of a late viremic phase. Wong and Freund (1951) injected mice and rabbits intracerebrally with either of two fixed strains, while the Russian Borodina (1959) used three street strains in mice. Heart blood collected within 2-1/2 hours (Wong and Freund) and 50 minutes to 54 hours (Borodina) after the intracerebral inoculation produced rabies in recipient mice. Wong and Freund also found virus in one rabbit's ear vein blood sample three hours after inoculation.

The mechanism by which the rabies virus reached the blood stream after intracerebral inoculation was not known (Wong and Freund). Either the virus reached the blood via the cerebrospinal fluid, or it entered the blood vessels damaged by the injection. Wong and Freund claimed that the first possibility was supported by the findings of Schaeffer and Muckenfuss (1940) on the distribution of India ink following intracerebral injection in monkeys.

The presence of rabies virus or antigen in blood cells has been allegedly detected by some workers. Frye and Enright (1964) observed acidophilic inclusions resembling Negri bodies in roughly 0.5-1.0 per cent of peripheral erythrocytes of mice intramuscularly injected with a coyote rabies strain. Baratawidjaja et al. (1965), as cited by Johnson (1971), found rabies virus antigen in circulating leucocytes of rabbits

intramuscularly inoculated with a porcine kidney-adapted strain of virus. Recently, a group of Russian investigators (Gorshunova et al., 1976) reported Babes-Negri bodies in erythrocytes of rabies-infected mice.

The route of rabies inoculation could have been a factor in the occurrence of viremia. Reagan et al. (1954) infected young dogs with rabies by rectal instillation and observed virus-like particles or inclusion-like bodies in electron micrographs of the infected dogs' erythrocytes. These were seen in the 90-hour post-inoculation blood sample. These workers concluded that the virus was present in the blood during this 90-hour period even when the puppies showed no signs of rabies during a 30-day observation.

Fischman and Ward (1968) observed rapidity of the course of rabies following oral infection and presence of virus in various non-nervous organs, both of which "point to a blood-borne infection".

Correa-Giron et al. (1970), although they did not test the blood of mice orally infected with rabies, noted that the presence of virus in the spleen at one hour after oral infection suggested viremia.

Hronovsky and Benda (1969), while studying the pathogenesis of inhalation rabies in suckling guinea pigs, found early and regular rabies immunofluorescence in the kidneys. They think that this "suggests a hematogenous infection... by virus resorbed into the blood stream from the sites of its primary multiplication in the nasal region."

Some evidence for rabies viremia came from the study

of Debbie and Trimarchi (1970). In studying the pantropism of the virus in naturally rabid foxes by the fluorescence test, they found rabies antigen in a wide range of non-nervous tissues. Significantly, the venous endothelial layer of one fox's adrenal gland was positive for rabies antigen. They considered this finding suggestive of viremia during some portion of the disease.

MATERIALS AND METHODS

MICE

A total of 490 weanling (three weeks old) white mice were used.

One hundred and fifty CF-1 mice[a], of either sex, comprised the experimental animals. These were randomly divided into three groups, 50 mice each, according to the route of rabies inoculation.

Two hundred and sixty mice, of either sex, were used for rabies virus titration and most as recipient mice in the test for viremia by mouse inoculation.

Additional 80 mice, used in initial titration studies and in preparing normal and infected mouse brain suspensions (for dilution of the rabies conjugate), came from the Kansas State University Diagnostic Laboratory's animal breeding colony.

All the mice, five per cage, were housed and managed by the K.S.U. Animal Research Facility during this research.

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[a]Charles River Breeding Laboratories, 252 Ballardvale St., Wilmington, Mass. 01887

RABIES VIRUS

Two skunk rabies isolates, #2030 and #5072, were provided by the K.S.U. Diagnostic Laboratory. These isolates were received as 20 per cent salivary gland suspensions with intracerebral MLD50 titers of $10^{5.6}$ and $10^{6.4}$ for #2030 and #5072, respectively, per 0.03 gm of gland.

Five rabid skunk heads were provided earlier by the same source. These were used in an initial attempt to select the infectious skunk salivary gland.

All isolates and infectious materials were stored at -70°C.

QUANTAL TITRATION

Isolates #2030 and #5072 were titered to check the LD50. As both isolates had been previously titered, only $10^{-4.5}$, 10^{-5}, $10^{-5.5}$ and 10^{-6} dilutions of #2030 and $10^{-5.5}$, 10^{-6}, $10^{-6.5}$ and 10^{-7} dilutions of #5072 in sterilized phosphate buffered saline (PBS, pH 7.6) with 10 per cent normal rabbit serum and 500 I.U. of penicillin and 1,500 I.U. of streptomycin antibiotics[b] per ml of diluent were used. Each dilution was inoculated intracerebrally into five weanling CF-1 mice, using 0.03 ml of the dilution per mouse. The techniques of intracerebral inoculation (see Appendix 1) was according to the method described by Koprowski (1973) and Atanasiu (1975) for mouse inoculation test (MIT), as routinely done in rabies diagnostic examination.

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[b] Pfizerpenstrep, Pfizer Laboratories Division, Pfizer Inc., New York, New York 10017

Inoculated mice were observed for at least 45 days. Based on the recorded mortalities and survivals, the MLD50 was calculated (Appendix 2) by the Reed-Muench method (Lorenz and Bogel, 1973).

INOCULATION OF EXPERIMENTAL MICE

Skunk salivary gland isolate #5072 was selected for inoculation because of its high titer, $10^{6.4}$ MLD50's. A 10^{-3} dilution of the gland was made as in the titration, and the volume administered was: 0.05 ml by droplet into each mouse's nares, intranasal group; 0.05 ml into the right gastrocnemius of each mouse, intramuscular group; and 0.50 ml by stomach tube per mouse, oral group. Forty mice per group were inoculated with the diluted salivary gland suspension, while 10 control mice in each group were given the same amount of diluent only (PBS with normal serum and antibiotics). All mice, including controls, were anesthetized by inhalation of ether[c] to facilitate inoculation. Disposable, glass, half-ml intradermal syringes[d] with 26-gauge, 3/8-inch long needle were used for intramuscular and intranasal administration. Intranasally, the inoculum was instilled by droplets into the outer nares, allowing the mouse to inhale the inoculum droplet by droplet. For oral administration, the needle was replaced with a two-inch-long piece of plastic tubing which fit well into the nozzle of the syringe. All group inoculations were completed within one

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[c] Ethyl ether, E.R. Squibb & Sons, Inc., Princeton, N.J. 08540

[d] Glaspak, Becton, Dickinson and Co., Rutherford, N.J.

hour. The inoculum was kept cold by holding the rabies suspension in an iced container.

BLOOD AND TISSUE SAMPLING

At 1, 3, 6 and 13 hours, and daily up to 21 days after inoculation, blood samples for viremia studies were taken from two mice drawn randomly from each rabies-inoculated group. Later, sick mice were preferred. Blood was drawn peripherally from the clipped tail of anesthetized mice. Two heparinized (with minimum of 3 U.S.P. units of anticoagulant activity) 75-mm-long microcapillary pipette[e] were used to draw approximately 0.1 ml of blood from each mouse. Blood drawn in the pipettes were then aspirated into a disposable syringe[d], pooling samples from mice of the same inoculation group and of the same sampling time. Blood from sick mice was not pooled; instead, these were kept in individual syringes. All syringes were immediately stored at -70°C until used in the intracerebral MIT.

Blood smears were also made on clean FA microscope slides[f] with a frosted end and two etched circles. The smears were air-dried and fixed in cold acetone[g] (-60°C) for an hour. Fixed smears were stored at -60°C until by immunofluorescence.

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[e] Microhematocrit capillary tubes, heparinized, Dade Division, American Hospital Supply Corp., Miami, Florida. Distributed by Scientific Products, General Offices, Evanston, Ill.

[f] FA Microscope slides, Dade Division, Division of American Hospital Supply Corp. Distributed by Scientific Products.

[g] Acetone, Spectranalyzed, Fisher Scientific C., Chemical Mfg. Division, Fair Lawn, N.J. 07410

To study the tissue distribution of the virus, impressions of brain, liver, lungs, heart, kidney, spleen, and gastrocnemius (left muscle for intramuscular group) were made from dead or dying mice. Tissue specimens were taken as aseptically as possible, using separate instruments for each mouse and cleaning the instruments before use on each organ of the same mouse. Impressions were made in the manner described by Tierkel (1973). A piece of an organ was placed on a clean wooden togue-depressor, cut surface up. A clean FA slide[f] was then touched against the cut surface, pressing gently the etched areas of the slide against the exposed surface of the tissue. After air-drying the impressions, they were fixed in cold acetone and stored in the same manner as the blood smears.

MOUSE INOCULATION TEST (MIT)

After thawing, the hemolyzed blood was inoculated directly, using the same syringe used for storing the blood, into anesthetized weanling mice. One or two CF-1 mice were inoculated intracerebrally per sample (at least three mice per group per sampling), injecting 0.03 to 0.04 ml of whole blood per mouse. Inoculation was done in the standard manner (Appendix 1). Inoculated mice were observed daily for at least 55 days for signs of rabies.

FLUORESCENT RABIES ANTIBODY TEST (FRAT)

The direct fluorescent rabies antibody test described by Dean and Abelseth (1973) and Kissling (1975) was applied to the blood smears and tissue impressions.

Adsorbing rabid (CVS-infected) and normal mouse brain

suspensions were prepared (Appendix 3) according to the procedure outlined by Howard (1976), with minor modification. These 20 per cent suspensions of mouse brains were used for diluting the rabies conjugate[h] into the final dilution used for staining.

Previously prepared slides (acetone-fixed) were stained with normal-mouse-brain-diluted conjugate in one marked area of the slide and with CVS-brain-diluted conjugate in another marked area. Rabies-positive control slides (infected mouse brains) and negative control slides (blood smears from control mice) were similarly stained.

The standard procedure of staining (Appendix 4) was followed as outlined (Anon., 1978), with very little modification.

Stained slides were examined under the UV-microscope[i] equipped with standard attachments (darkfield condenser, mercury vapor lamp, filters).

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h BBL Anti-Rabies globulin, fluorescein labeled, Division of Becton, Dickinson and Co., Cockeysville, Maryland 21030

i Ortholux FA scope and accessories, Leitz Wetzler, Germany. E. Leitz, Inc., Rockleigh, N.J. 07647

RESULTS

COMPARATIVE INFECTIVITY (Table 1 and Figure 1)

The highest mortality was recorded in the intranasally inoculated group. Of the 40 mice in this group, 37 (92%) died in an average of 18.4 days from the time of nasal instillation of the rabies inoculum.

Intramuscularly inoculated mice had 11 deaths, or 28 per cent mortality. These averaged 23.6 days from exposure to death.

Orally inoculated mice had the lowest mortality. Two of 40 died (5%), one on the 18th day and the other on the 20th day post-inoculation.

Table 1. Infectivity of non-passaged skunk rabies virus isolate in weanling mice by different routes of inoculation.

Route of Inoculation	Dose Per Mouse	Mortality Rate	Days From Exposure To Death
Oral	0.5 ml*	5% (2/40)**	19 (18, 20)
Nasal	0.05 ml*	92% (37/40)**	18.4 (13-22)
Intramuscular	0.05 ml*	28.5%(11/40)**	23.6 (18-27)
Intracerebral***	0.03 ml*	100% (5/5)	13 (12-15)
Control****	0.05 or 0.5 ml	0% (0/30)	---

*A 10^{-3} dilution of skunk salivary gland (#5072) with an intracerebral MLD50 titer of $10^{6.4}$ per 0.03 ml of gland

**Highly significant differences by Chi-square test at alpha=.05

***Data from titration of #5072

****Ten control mice per group given same amount of diluent via the same inoculation route as rabies-injected mice

For background comparison, all five mice intracerebrally inoculated with the same dose in the titration of the isolate died in an average of 13 days.

Tremors, ruffling of the fur, hunched-back appearance and, shortly later, prostration and paralysis were seen in the mice dying of rabies from the intracerebral, nasal, or oral inoculation of the virus. Mice dying from the intramuscular rabies infection showed irritability, listlessness and hyperesthesia followed by sudden death. The brains of these mice were all positive by the FRAT, confirming the clinical diagnosis of rabies.

None of the 30 control mice (10 per group), similarly inoculated but with diluent only, died during the 45-day observation period.

The differences in mortalities between the inoculation groups are statistically significant by the Chi-square test at an alpha level of .05.

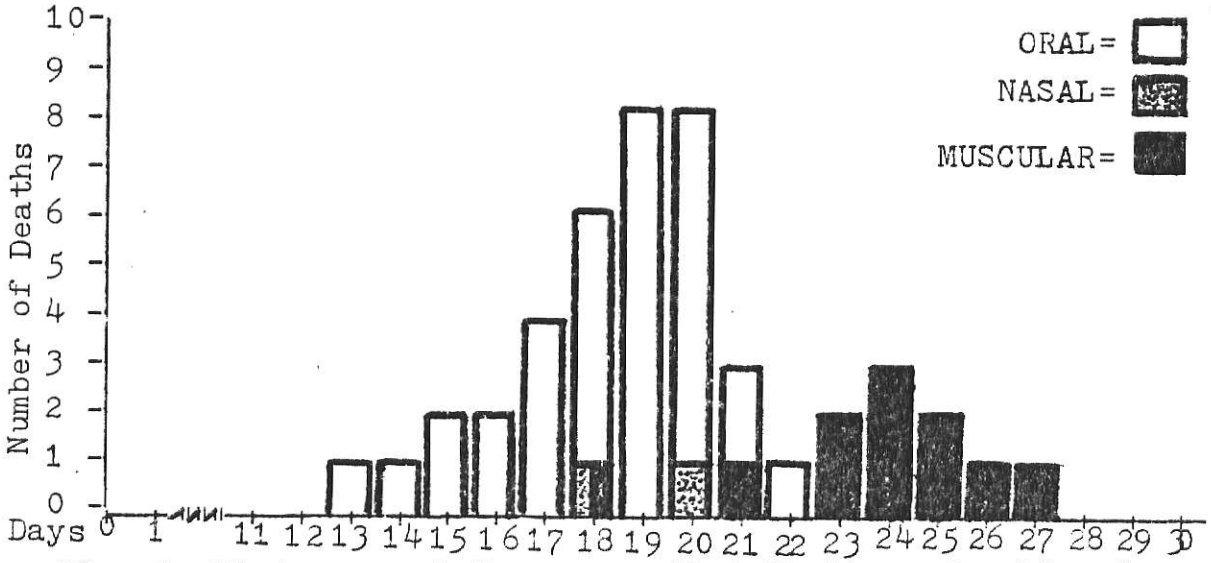

Fig. 1. Histogram of frequency distribution of rabies in mice according to route of administration of skunk rabies virus isolate #5072 and occurrence of death.

RABIES VIREMIA STUDY

Neither the MIT nor the FRAT gave positive results (Appendix 5). Blood from clinically rabid mice was negative by both tests.

One recipient mouse died from an apprently generalized skin reaction to injected blood, and its brain was FRAT-negative; all of the other mice used in the MIT survived in an observation period of 55 to 83 days.

No distinctive fluorescence could be found in any of the blood smears from the inoculation groups. Most of the erythrocytes were disrupted and, as in the control blood smears, exhibited a lot of nonspecific fluorescence.

RABIES TISSUE DISTRIBUTION (Table 2)

The organ, or tissue, distribution of the rabies virus in 22 mice dead or dying from rabies following either route of inoculation is summarized in Table 2. Ten came from each of the nasally and intramuscularly inoculated groups and two belonged to the oral group. The brains of all 22 mice were positive by the FRAT, and the other six organs or tissues selected for this study were positive in most cases. The least positive organs were the liver of the nasally inoculated mice (4/10) and the spleen of the intramuscularly infected mice (3/10). Four of the nasally inoculated and three of the intramuscularly infected mice were negative for both liver and spleen (Appendix 6). Most of these mice had a shorter incubation period than mice with a greater organ distribution. The rest of the organs or tissues (gastroc-

nemius muscle, heart, kidney, and lung) were positive in at least 70 per cent of the specimens regardless of the route of infection. No mouse was found negative for more than three organs or tissues examined.

Table 2. Distribution of rabies virus on selected organs of dead or moribund mice by fluorescent rabies antibody test (FRAT).

Organ Tested	Number of Organ Positive/Total No. Tested Per Gp.		
	Oral Group	Nasal Group	Intramuscular
Brain	2/2	10/10	10/10
Heart	2/2	7/10	8/10
Lung	2/2	9/10	8/10
Liver	1/2	4/10	5/10
Kidney	2/2	8/10	9/10
Spleen	1/2	5/10	3/10
Gastrocnemius	2/2	9/10	9/10

Overall, the route of rabies inoculation did not influence the distribution of the virus among the organs or tissues examined.

DISCUSSION

The results demonstrated that:

1. nasal instillation was more infective than either intramuscular (rear leg) or oral (stomach entubation) administration of the virus isolate in mice;
2. there was no demonstrable rabies viremia, at any stage, following these inoculations; and
3. widespread centrifugal dissemination of the virus occurred at the terminal stage of the disease.

The infectivity of intranasal rabies inoculation has been shown in previous studies in mice and other animals with either fixed or street strains (Atanasiu, 1965; Hronovsky and Benda, 1969; Fischman and Schaeffer, 1971; Hronovsky, 1971; Charlton and Casey, 1979 a and b). Most of this research and those who studied oral, or ingestion, rabies (Fischman, 1969; Correa-Giron et al., 1970) were primarily devoted to the pathogenetic aspects of these types of infection. No comparison of the infectivity between the three recognized routes of rabies infection--intramuscular, oral, and nasal--was made by these workers, except by Charlton and Casey who compared the oral and nasal infectivities of various rabies isolates.

Charlton and Casey (1979a) showed that mice were more susceptible to intranasal infection than oral administration, the latter either by moistened pledget in mouth, stomach entubation, or feeding infected mouse brain free-choice. Results of the present study corroborated the greater susceptibi-

lity of mice to nasal inoculation than to stomach entubation and, in addition, to intramuscular injection into the gastrocnemius. Stomach entubation produced only 5% mortality in this study inspite of a dose ten times that of the intranasal or intramuscular infections. Apparently, a very high dose is necessary to produce oral infection, as previously reported. Fischman and Ward (1968) used 83,000 intracerebral MLD50's for neonatal mice and 666,000 for adult mice of the fixed strain CVS to produce a high rate of infection. Correa-Giron et al. (1970) used 100 million MLD50's of the CVS and 320,000 MLD50's of a bovine strain in producing about equal mortality rates (34%) in mice. Bell and Moore (1971) infected less than 6% of mice inoculated with almost 20 million MLD50 doses of the fixed virus. The oral dose used in this study was roughtly 70,000 MLD50's of the skunk isolate. One-tenth of this dose produced higher mortalities in mice inoculated nasally (92%) or intramuscularly (28.5%), clearly showing the inferiority of stomach entubation in producing rabies infection in mice. A large proportion of the virions either failed to adsorb to the alimentary tract or were inactivated by the acid secretions of the stomach. High concentrations of the virus or large volume of infectious material, therefore, may be required to induce rabies by this route.

Dosage may not be the only factor involved in the lower infectivity of oral inoculation observed in this study. Serokowa (1969), as cited by Charlton and Casey (1979a), suggested that oral infection could actually be due to adsorption of virus in the nasal mucosa. Hence, it could be that the two

rabies deaths from stomach entubation in the study were actually the result of the inoculum getting into the nasal mucosa in the process of inoculation, or as a consequence of regurgitation. The incubation period and clinical signs of these mice were similar to nasally infected mice.

The pathogenesis of nasal rabies is still unknown. According to Hronovsky (1971), "it stands close to cerebral infection" because of the proximity of the site to the brain and the richness of enervation from this organ. Again, the short incubation period observed and the paralytic type of rabies that resulted from nasal infection, which were similar to rabies by intracerebral route, seemed to support this view.

Comparison with the intramuscular route in this study was limited to the gastrocnemius site and results could not be applied to other muscular areas. It is known that the site of inoculation influences the length of incubation period of rabies (Johnson, 1971; Debbie, 1974). Probably, a shorter incubation period and higher mortalities might have been observed if intramuscular injection was closer to the brain. However, the inoculated groups of mice were kept for 85 days of observation in this study so that the chance of missing mice with long incubation periods was less than if they had been observed for a shorter time.

The relatively low mortality rate from intramuscular inoculation (compared to intranasal) was unexpected since this route, which approximates the bite--the principal mode of rabies transmission, has been routinely used in numerous

studies of rabies by other workers. The dose of 7,000 MLD50's was thought to be adequate to kill most mice so-inoculated. The isolate used might have been less invasive, as there are reports that strains may differ in invasiveness by one or more routes (Serokowa, 1969; Correa-Giron et al., 1970; Bell and Moore, 1971; Baer et al., 1977). However, Charlton and Casey (1979a) reported no difference in infectivity, by oral and intranasal, between the various strains they tested, including a fixed one (CVS). In the present study, an initial attempt was made to select a highly invasive skunk isolate by inoculating four mice each with 0.1% suspension intracerebrally and 0.01% intramuscularly of salivary gland from five rabid skunks diagnosed by the K.S.U. rabies diagnostic laboratory. While all mice inoculated intracerebrally with the 10^{-3} dilution died, those injected intramuscularly in the gastrocnemius with a 10^{-2} dilution of the same material survived, with only one death out of the 16 inoculated. These mice were observed for almost three months, with six mice giving birth to litters during the period. The failure to infect mice intramuscularly, even in the presence of stress (pregnancy), was the reason why none of these isolates were used in latter inoculations. Retrospectively, however, most of these isolates could have produced rabies intranasally although they might have failed to infect intramuscularly or orally with the same dilution used in the inoculations. A high titer maybe required to produce intramuscular infections, but less was needed to nasally infect mice.

Viremia. No direct evidence for rabies viremia was gathered in this study, despite the application of the two sensitive tests for rabies, the MIT and the FRAT (Kissling, 1975). If viremia does occur following rabies infection, the chance of its being detected should be greatest in the intranasally inoculated group and least in the orally infected mice, chance being based on the mortality rate resulting from the inoculations. The chance of detecting viremia in each inoculation group was increased when blood samples were taken from mice already showing rabid signs, although this represented the stage when the virus had already invaded the central nervous system. Further, the presence of virus in most of the organs selected for tissue distribution study by FRAT favored finding of rabies virus or antigen in the blood, if this was really being shed from extra-neural site(s). Extra-neural replication of the rabies virus is now recognized, being best exemplified by the salivary gland's higher titer than the brain in most cases (Dierks, 1975). The drawing and testing of blood from already rabid mice with generalized organ distribution of the virus, therefore, enhanced detection of viremia if ever this did occur.

The failure to positively detect viremia agrees with the findings of Fischman and Schaeffer (1971) and Murphy et al. (1973) in intranasally infected mice and those of Schindler (1961), Baer et al. (1965), and Murphy et al. (1973) in intramuscularly inoculated animals (mouse, rat, or Syrian hamster). The fact that virus could not be demonstrated at any time in the blood following either of three routes of inoculation

tends to support the idea that viremia in rabies is an exception rather than a rule. Probably, blood-borne infection or dissemination does occur in animals of high rabies susceptibility, as suggested by Dean et al. (1963). The inability to demonstrate viremia in this study was not conclusive, particularly in the orally inoculated group. Higher infectivity rates in various species of animals and various strains of rabies virus may be needed to fully investigate the question of rabies viremia.

Organ Distribution. "There is hardly any organ which at one time or another cannot be shown to yield rabies virus or antigen if a careful enough search is made (Schneider, 1975)." This statement regarding centrifugal spread of the virus from the central nervous system was supported by the finding of rabies antigen in most of the organs tested in this study, and also by a number of previous reports that the virus was isolated from peripheral nerves and non-nervous organs (Schneider, 1975). Much of the spread in various organs could be by the neural pathway, although extra-neural multiplication sites could have existed. However, in the present study, whether the virus was reproducing in the glandular tissue, incidental to the nerve supply, or was associated with other cells within these tissues, could not be determined. Localization of antigen in the tissue impressions could not be determined definitively. However, organs with less nervous tissue supply (spleen and liver) were the least positive relative to the other selected organs. It seems that the route of inoculation had little effect on

the extent of dissemination of the virus; rather, it is the extent and nature of enervation that involves organs other than the central nervous system. Schneider and Hamann (1969) pointed out that the length of nervous connection to the brain and the abundance or nerves of the organs determined the time and intensity of their infection, the infectious dose being considered to be standardized. This seems to have been the case in this study. Lastly, the presence of the virus in the brain at the time other (non-brain) organs were FRAT-positive indicated a centrifugal spread from the central nervous system through neural pathways, since we were unable to demonstrate a viremia in this study.

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BIBLIOGRAPHY

Anon. 1978. Rabies virus. In: Manual of Standardized Methods for Veterinary Microbiology, G.E. Cottral (ed.), 1st edtion. London and Ithaca: University Press. pp. 301-306.

Atanasiu, P. 1965. Transmission de la rage par la voie aerienne aux animaux de laboratoire. International Symposium on Rabies, Talloires, 1965. Symp. Ser. Immunobiol. Stand. 1:159-166.

Atanasiu, P. 1975. Animal inoculation and the Negri body. In: The Natural History Of Rabies, G.M. Baer (ed.), 1st edition, volume 1. San Francisco and New York: Academic Press. Chapter 19, pp. 373-400.

Baer, G.M. 1975. Pathogenesis to the central nervous system. In: The Natural History of Rabies, G.M. Baer (ed.), 1st edition, volume 1. San Francisco and New York: Academic Press. Chapter 10, pp. 181-198.

Baer, G.M., Cleary, W.F., Diaz, A.M., and D.F. Perl. 1977. Characteristics of 11 rabies virus isolates in mice: titers and relative invasiveness of virus, incubation period of infection, and survival of mice with sequelae. J. Infect. Dis. 136(3):336-345.

Baer, G.M., Shathaveerappa, T.R., and G.H. Bourne. 1965. Studies on the pathogenesis of fixed rabies virus in rats. Bull. Wld. Hlth. Org. 33:783-794.

Baratawidjaja, R.K., Morrisey, L.P., and N.A. Labsoffsky. 1965. Demonstration of vaccinia, lymphocytic choriomeningitis and rabies viruses in the leucocytes of experimentally infected animals. Arch. Ges. Virusforsch. 17: 273-284.

Bell, J.F., and G.J. Moore. 1971. Susceptibility of carnivora to rabies virus administered orally. Am. J. Epidemiol. 93(3):176-182.

Borodina, T.A. 1959. A study on viremia in experimental rabies. Vopr. Virusol. 4(2):96-100.

Burne, J.C. 1970. Viremia in rabies. (Letter) Lancet 1: 195-196.

Center for Disease Control. 1977. Rabies in a laboratory worker--New York. Morbid. Mortal. Wkly. Rep. 26(22): 183-184.

Center for Disease Control. 1979. Human-to-human transmission of rabies by corneal transplant--Idaho. Morbid. Mortal. Wkly. Rep. 28:109-111. (Also in Vet. Pub. Hlth. Notes, April, 1979:1-3.)

Charlton, K.M., and G.A. Casey. 1979a. Experimental oral and nasal transmission of rabies virus in mice. Can. J. Comp. Med. 43(1):10-15.

Charlton, K.M., and G.A. Casey. 1979b. Experimental rabies in skunks: oral, nasal, tracheal and intestinal exposure. Can. J. Comp. Med. 43(2):168-172.

Constantine, D.G. 1962. Rabies transmission by nonbite route. Vet. Pub. Hlth. Rep. 77:287-289.

Constantine, D.G. 1967. Rabies transmission by air in bat caves. U.S. Pub. Hlth. Ser. 1671. Government Printing Office, Washington, D.C.

Constantine, D.G. 1971. Bat rabies: current knowledge and future research. In: Rabies, Y. Nagano and F.M. Davenport (eds.), 1st edition. Tokyo: University Park Press. pp. 253-262.

Correa-Giron, E.P., Allen, R., and S.E. Sulkin. 1970. The infectivity and pathogenesis of rabies virus administered orally. Am. J. Epidemiol. 93(3):176-182.

Dean, D.J., Evans, W.M., and R.C. McClure. 1963. Pathogenesis of rabies. Bull. Wld. Hlth. Org. 29:803-811.

Dean, D.J., and M.K. Abelseth. 1973. The fluorescent antibody test. In: Laboratory Techniques in Rabies, M.M. Kaplan and H. Koprowski (eds.), 3rd edition. Geneva: W.H.O. Press. Chapter 6, pp. 73-84.

Debbie, J.G. 1974. Rabies. Prog. Med. Virol. 18:241-256.

Debbie, J.G., and C.V. Trimarchi. 1970. Pantropism of rabies virus in free-ranging rabid red fox Vulpes fulva. J. Wildl. Dis. 6:500-505.

Dierks, R.E. 1975. Electron microscopy of extraneural rabies infection. In: The Natural History of Rabies, G.M. Baer (ed.), 1st edition, volume 1. San Francisco and New York: Academic Press. Chapter 15, pp. 303-318.

DiVestia and G. Zagari. 1887. Sur la transmission de la rage par voie nerveuse. Ann. Inst. Pasteur 3:237-248.

Fischman, H.R. 1969. Fluorescent antibody staining of rabies-infected tissues embedded in paraffin. Am. J. Vet. Res. 30(7):1213-1221.

Fischman, H.R., and F.E. Ward III. 1968. Oral transmission of rabies virus in experimental animals. Am. J. Epidemiol. 88(1):132-138.

Fischman, H.R., and M. Schaeffer. 1971. Pathogenesis of experimental rabies as revealed by immunofluorescence. In: Defined Immunofluorescent Staining, E.H. Beutner (ed.), 1st edition, volume 177. New York: New York Academy of Sciences. pp. 78-93.

Frederickson, L.E., and L. Thomas. 1965. Relationship of fox rabies to caves. Pub. Hlth. Rep. 80:495-500.

Frye, F.L., and J.B. Enright. 1964. Observations of erythrocytic inclusion bodies from mice infected with rabies virus. Proc. Soc. Exptl. Biol. Med. 115(3):689-691.

Gorshunova, L.P., Kantorovich, R.A., and G.V. Korbut. 1976. Detection of Babes-Negri bodies in blood erythrocytes of rabid white mice. Vopr. Virusol. 76(2):179-182.

Hagan, W., and D.W. Bruner. 1960. Rabies. In: The Infectious Diseases of Domestic Animals. Ithaca, New York: Comstock Publishing Co. Chapter 44.

Howard, D.R. 1976. Detection of rabies virus in selected tissues of naturally infected skunks. Proc. 19th Ann.

Meet. Am. Assoc. Vet. Lab. Diags. 1:265-278.

Houff, S.S., Burton, R.C., Wilson, R.W., Henson, T.E., London, W.T., Baer, G.M., Anderson, L.J., Winkler, W.G., Madden, D.L., and J.L. Sever. 1979. Human-to-human transmission of rabies virus by corneal transplant. New Engl. J. Med. 300:603-604.

Hronovsky, V. 1971. Immunofluorescence study on the pathogenesis of fixed rabies virus respiratory infection in suckling mice. Acta. Virol. 15(1):58-64.

Hronovsky, V., and R. Benda. 1969. Development of inhalation rabies infection in suckling guinea pigs. Acta. Virol. 13(3):198-202.

Hronovsky, V., and R. Benda. 1969. Experimental inhalation infection of laboratory rodents with rabies virus. Acta. Virol. 13(3):193-197.

Humphrey, G.L., Kemp, G.E., and E.G. Wood. 1960. A fatal case of rabies in a woman bitten by an insectivorous bat. Pub. Hlth. Rep. 75:317-325.

Hutyra, F., Marek, J., and R. Manninger. 1949. Rabies. In: Special Pathology and Therapeutics of the Diseases of Domestic Animals. Section IV, volume 1. London: Balleire, Tindall and Cox.

Irons, J.V., Eads, R.B., Grimes, J.E., and A. Conklin. 1957. The public health importance of bats. Texas Rep. Biol. Med. 15:292-298.

Johnson, H.N. 1959. The role of the spotted skunk in rabies. Proc. 63rd Ann. Meet. U.S.L.S.A. 1:267-274.

Johnson, H.N. 1965. Rabies. In: Viral and Rickettsial Infections in Man, F.L. Horsfall and I. Tamm (eds.), 4th edition. Philadelphia, Pa.: Lippincott and Co.

Johnson, R.T. 1971. The pathogenesis of experimental rabies. In: Rabies, Y. Nagano and F.M. Davenport (eds.), 1st edition. Tokyo: University Park Place.

Kaplan, C. 1977. The world problem. In: Rabies--The Facts, C. Kaplan (ed.), 1st edition. Oxford, England: Oxford University Press. pp. 1-21.

Kauker, E. 1967. Rabies in Central Europe from 1953-1966. Report of a sessional meeting of the Heidelberg Academy of Sciences, 1966, 4th meeting. Abst. in Trop. Dis. Bull. 64:615-619.

Kelser, R.A. 1955. Rabies. In: Diseases Transmitted from Animals to Man, T.G. Hull (ed.), 4th edition. Springfield, Ill.: Thomas Publishing Co. Chapter 9.

Kissling, R.E. 1975. The fluorescent antibody test in rabies. In: The Natural History of Rabies, G.M. Baer (ed.), 1st edition, volume 1. San Francisco and New York: Academic Press. Chapter 20, pp. 401-416.

Kitselman, C.H., and A.K. Mital. 1967. The pathogenesis of rabies in the rat. Can. J. Comp. Med. Vet. Sci. 31:122-124.

Koprowski, H. 1973. The mouse inoculation test. In: Laboratory Techniques in Rabies, M.M. Kaplan and H. Koprowski (eds.), 3rd edition. Geneva: W.H.O. Press. Chapter 7, pp. 85-93.

Kovalev, A.A., Sedov, V.A., and A.S. Shasherko. 1971. Experimental studies of certain ways in which rabies is transferred. Proc. 19th Wld. Vet. Congr., Mexico City 2:711-712.

Krause, W.W. 1957. Kritische experimentelle studien uber die prufung von tollwutimpfstoffen, die pathogenese des lyssa und das geschehen in des inkubations periode. II. Mitteilung die pathogenese der toolwut und die prakitische wertung der Wutschutzbehandlung. Zbl. Bakt. I (Orig.) 167:481-503.

Krause, W.W. 1966. The pathogenesis of rabies. Symp. Ser. Immunobiol. Standard. 1:153-165.

Lorenz, R.J., and K. Bogel. 1973. Calculation of titers and

their significance: B. Method of Reed and Muench. In: Laboratory Techniques in Rabies, M.M. Kaplan and H. Koprowski (eds.), 3rd edition. Geneva: W.H.O. Press. pp. 329-332.

Matsamuto, S. 1970. Rabies virus. In: Advances in Virus Research, K.M. Smith, M.A. Lauffer and F. Beng (eds.). New York and London: Academic Press. pp. 257-301.

Murphy, F.A. 1977. Rabies pathogenesis. Arch. Virol. 54(4): 279-295.

Murphy, F.A., Bauer, S.P., Harrison, A.K., and W.C. Winn, Jr. 1973. Comparative pathogenesis of rabies and rabies-like viruses: viral infection and transit from inoculation site to the central nervous system. Lab. Invest. 28(3): 361-376.

Murphy, F.A., Harrison, A.K., Winn,Jr., W.C., and S.P. Bauer. 1973. Comparative pathogenesis of rabies and rabies-like viruses: infection of the central nervous system and centrifugal spread of virus to peripheral tissues. Lab. Invest. 29(1):1-16.

National Research Council Subcommittee on Rabies. 1973. The disease. In: Control of Rabies. Washington, D.C.: National Academy of Sciences Printing and Publishing Office. pp. 1-4.

Noble, E.R., and G.A. Noble. (eds.) Parasitology: The Biology of Animal Parasites, 4th edition. Philadelphia: Lea and Febiger. p. 391.

Parker, R.L. 1975. Rabies in skunks. In: The Natural History of Rabies, G.M. Baer (ed.), 1st edition, volume 2. San Francisco and New York: Academic Press. Chapter 3, pp. 41-52.

Pasteur, L., Chamberland, S., Roux, E., and G. Thuillier. 1881. C.R. Acad. Sci. 92:1259. Cited by Baer, G.M., in The Natural History of Rabies, 1st edition, volume 1, p. 181.

Ramsden, R.O., and D.H. Johnston. 1975. Studies on the oral infectivity of rabies virus in carnivores. J. Wildl. Dis. 11(2):318-324.

Reagan, R.L., Yancy, F.S., Chang, S.C., and A.L. Brueckner. 1954. Transmission of street rabies virus strain (V308) to suckling hamsters during lactation. Proc. Soc. Exp. Biol. Med. 90:301-302.

Remlinger, R. 1908. Transmission de la rage a la souris par ingestion. C. R. Seanc. Soc. Biol., Paris, 31:385-386.

Schaeffer, M., and R.S. Muckenfuss. 1940. Experimental Poliomyelitis, 1st edition. Philadelphia: Science Press Printing Co. Cited by Wong, D.H., and J. Freund. 1951. Proc. Soc. Exp. Biol. 76:717-720.

Schindler, R. 1961. Studies on the pathogenesis of rabies. Bull. Wld. Hlth. Org. (W.H.O.) 25:119-126.

Schindler, R. 1966. Pathogenesis of rabies infection. International Symposium on Rabies, Talloires, France, 1965. Symp. Ser. Immunobiol. Standard. 1:147-155.

Schneider, L.G. 1969. The pathogenesis of rabies in mice. I. The spread of the virus from the site of infection towards the central nervous system. Zbl. Bakt. Parasit. Infek. Hyg. (Abst. in English) 211:281-308.

Schneider, L.G. 1969. The pathogenesis of rabies in mice: II. The spread of virus in the central nervous system. Zbl. Bakt. Parasit. Infekt. Hyg. (Abst. in English) 212: 1-13.

Scneider, L.G. 1975. Spread of virus from the central nervous system. In: The Natural History of Rabies, G.M. Baer (ed.), 1st edition, volume 1. San Francisco and New York: Academic Press. Chapter 14, pp. 273-301.

Schneider, L.G., and I. Hamann. 1969. Die pathogenese der tollwut bei der mau. III. Die zentrifugale virusausbreitung und de virusgeneralisierung im organismus. Zbl. Bakt. Parasit. Infekt. Hyg.(Abst. in English) 212:13-21.

Serokowa, D. 1969. Food-borne infection with rabies virus under experimental conditions. Epidemiol. Rev. 23:122-134.

Shashenko, A.S., and N.A. Kovalev. 1971. Distribution of rabies virus in the body and its isolation. Veterinariya (Moskva) 47:44-46.

Soave, O.A. 1966. Transmission of rabies to mice by ingestion of infected tissue. Am. J. Vet. Res. 27(16):44-46.

Steele, J.H. 1975. History of rabies. In: The Natural History of Rabies, G.M. Baer (ed.), 1st edition, volume 1. San Francisco and New York: Academic Press. Chapter 1, pp. 1-32.

Sulkin, S.E., Kurtsch, P.H., Wallis, C., and R. Allen. 1957. The role of brown fat in the pathogenesis of rabies in insectivorous bats. Proc. Soc. Exp. Biol. Med. 96:461-469.

Tierkel, E.S. 1963. Rabies. In: Diseases Transmitted from Animals to Man, T.G. Hull (ed.). Springfield, Ill.: Thomas Publishing Co. Chapter 9.

Tierkel, E.S. 1971. Historical review of rabies in Asia. In: Rabies, Y. Nagano and F.M. Davenport (eds.), 1st edition. Tokyo: University Park Press. pp. 3-10.

Tierkel, S.E. 1973. Shipment of specimens and techniques for preparation of animal tissues. In: Laboratory Techniques in Rabies, M.M. Kaplan and H. Koprowski (eds.), 3rd edition. Geneva: W.H.O. Press. Chapter 3, pp. 29-40.

Vanag, K.A. 1978. Attempts to infect Rhipicephalus sanguineus (Latr.) ticks with the rabies virus. Med. Parazitol. (Mosk.) 47(3):47-50.

Waterhouse, R. 1970. "Report of the Committee of Inquiry on Rabies." HM Stationery Office, London. Cited by Winkler, W.G. 1975. In: The Natural History of Rabies, 1st edition, volume 2, p. 120.

West, G.P. 1973. Rabies in Animals and Man. New York: Arco Publishing Co., Inc. pp. 12-13.

Wiktor, T.J., and H.F. Clark. 1975. Growth of rabies virus in cell culture. In: The Natural History of Rabies, G. M. Baer (ed.), 1st edition, volume 1. San Francisco and New York: Academic Press. Chapter 9, pp. 155-179.

Winkler, W.G. 1968. Airborne rabies virus isolation. J. Wildl. Dis. 4:37-40.

Winkler, W.G., McKean, H., and D.B. Adams. 1972. (Unpublished data.) Cited by Winkler, W.G. 1975. In: The Natural History of Rabies, G.M. Baer (ed.), 1st edition, volume 2, p. 120.

Winkler, W.G., Baker, E.F.,Jr., and C.C. Hopkins. 1972. An outbreak of nonbite transmitted rabies in a laboratory animal colony. Am. J. Epidemiol. 95:267-277.

Winkler, W.G., Fashinell, T.R., Luffingwell, L., Howard, P., and J.P. Conomy. 1973. Rabies in a laboratory worker. J.A.V.M.A 226:1219-1221.

Winkler, W.G. 1975. Airborne rabies. In: The Natural History of Rabies, G.M. Baer (ed.), 1st edition, volume 2. San Francisco and New York: Academic Press. Chapter 7, pp. 115-121.

Wong, D.H., and J. Freund. 1951. Fixed rabies virus in blood following intracerebral inoculations into mice and rabbits. Proc. Soc. Exp. Biol. Med. 76:717-723.

A P P E N D I C E S

APPENDIX 1
Mouse Inoculation Test

The mice were anesthetized by inhalation of ether prior to inoculation. The anesthetized mouse was laid on its left side with its legs toward the inoculator. The left thumb was placed under the lower jaw and the index finger on the top of the skull, pulling the skin lightly from the head, thus fixing the mouse's head firmly, and taking care not to suffocate the mouse.

The syringe was held perpendicular to the head and the needle was pushed rapidly 1-2 mm through the brain case, about 2 mm from the frontal arch, in the direction of the median line. The inoculum (0.03 to 0.04 ml) was injected and the needle withdrawn.

Inoculated mice were checked shortly after inoculation and dead ones were replaced by others inoculated with the same specimen (skunk salivary gland suspension or mouse bloo).

All inoculations with the same specimen were completed within one hour.

Inoculated mice were observed for a period of at least 45 days. Brains of dead mice were tested by FRAT to confirm clinical diagnosis of rabies.

APPENDIX 2

Records of Deaths and Survivals And Computation of the MLD50's

Table 1. Results of intracerebral inoculation of dilutions of rabies isolate #2030 in weanling mice.

Dilution*	No. Dead/No. Inoculated	Post-Inoculation Days of Deaths	Average Time of Deaths
$10^{-4.5}$	4/5	17, 17; 19; 22	18.8 days
10^{-5}	4/5	19; 20; 22; 24	21 days
$10^{-5.5}$	2/5	22;26	24 days
10^{-6}	0/5	---	---

*0.03 ml per mouse

Calculation of the MLD50 of #2030 by the Reed-Muench method.

Dilution	No. Survived	No. Died	Cumulative Totals: Survived	Cumulative Totals: Died	Percentage Mortality
$10^{-4.5}$	1	4	1	10	10/11 (90%)
10^{-5}	1	4	2	6	6/8 (75%)
$10^{-5.5}$	3	2	5	2	2/7 (29%)
10^{-6}	5	0	10	0	0/10 (0%)

$$\text{Difference of Logarithm} = \frac{50\% - (\%\text{ mortality next below 50\%})}{\%\text{ mortality next above 50\%} - \%\text{ mortality next below 50\%}}$$

multiplied by

logarithm of dilution factor;

$$\text{Difference of Logarith} = \frac{50 - 29}{75 - 29} \times .45 = 0.2;$$

Subtracting the difference of logarithm, 0.2, from the reciprocal of the starting point dilution, 5.5: 5.5-0.2 = 5.3;

Hence, log (50% endpoint dilution) of #2030 = -5.3;

And 50% endpoint dilution = $10^{-5.3}$;

The reciprocal of this dilution is the MLD50 titer = $10^{5.3}$.

Table 2. Results of intracerebral inoculation of dilutions of rabies isolate #5072 in weanling mice.

Dilution*	No. Dead/No. Inoculated	Post-Inoculation Days of Deaths	Average Time of Deaths
10^{-3}**	5/5	12, 12; 13, 13; 15	13 days
$10^{-5.5}$	4/5	19; 21; 23; 24	21.7 days
10^{-6}	4/5	19; 22; 23; 31	23.7 days
$10^{-6.5}$	3/5	25; 26; 30	27 days
10^{-7}	0/5	---	---

*0.03 ml per mouse

**For comparing incubation periods of intracerebral and intramuscular inoculations; data for this dilution was excluded in the calculation of titer.

Calculation of the MLD50 titer of #5072 by the Reed-Muench method.

Dilution	No. Survived	No. Died	Cumulative Totals: Survived	Cumulative Totals: Died	Percentage Mortality
$10^{-5.5}$	1	4	11	1	11/12 (92%)
10^{-6}	1	4	7	2	7/9 (78%)
$10^{-6.5}$	2	3	3	4	3/7 (43%)
10^{-7}	5	0	0	9	0/9 (0%)

Difference of Logarithm= $\frac{50 - 43}{78 - 43} \times 0.45 = 0.090$;

Subtracting the difference of logarithm, 0.09, from the reciprocal of the starting point dilution, 6.5: 6.5 - 0.09 = 6.41;

Hence, log (50% endpoint dilution) = -6.4;

And 50% endpoint dilution = $10^{-6.4}$.

The reciprocal of this dilution is the MLD50 titer = $10^{6.4}$.

APPENDIX 3

Preparation of Adsorbing CVS-Infected And Normal Mouse Brain Suspensions And Dilution of Conjugate

A. 20% CVS II-Infected Mouse Brain Suspension (For Specificity Control):

A 10^{-3} dilution of the CVS fixed strain of rabies virus in 0.01 M phosphate buffered saline (pH 7.6) was made, and 0.02 ml of the dilution was inoculated intracerebrally into each of 30 mice. As the mice became paralyzed in 6 or 7 days, brains were harvested aseptically. To make the 20% brain suspension 4.0 gms of the harvested mouse brains, 3.2 ml of sterile horse serum, and 12 ml of phosphate buffered saline with 90 mg (0.75%) of bovine albumin fraction V and 2.0 mg merthiolate were placed in a grinder (mortar and pestle) and were mixed and ground very thoroughly for about 15 minutes. Ground material was transferred to a centrifuge tube and was centrifuged at 1,000 r.p.m. for 5 minutes. The supernatant was drawn off. This supernatant was used for making the final working dilution of the reconstituted lyophilized commercial conjugate, i.e., in making the highest dilution for optimal fluorescence determined beforehand in FRAT trials. The final conjugate-rabid mouse brain suspension was incubated for 15 minutes at 37°C in a water bath to insure complete reaction of the rabies antigen with labeled antibody. This adsorbed conjugate, the specificity control, was stored at -70°C until used.

B. Normal Mouse Brain 20% Suspension:

Brains were harvested directly from 30 healthy, normal (non-infected) weanling mice. The same procedures were followed in making the brain suspension and in the preparation of the corresponding conjugate working dilution as were in adsorbing CVS-infected brain suspension.

APPENDIX 4

Fluorescent Rabies Antibody Test (FRAT)

Blood smears or tissue impressions were air-dried. The slides were placed in a Coplin jar containing cold acetone (-60°C) for 1 to 2 hours to fix the smears or impressions. After fixation, the slides were removed from the acetone and air-dried. These slides were either stored at -60°C for later staining, or immediately used in the FRAT. In staining, two suitable uniformly thin areas of the impression or smear, each approximately 3 cm long and situated within or by the etched circles of the slide, were marked with a wax marking pencil. These slides, including previously prepared controls similarly marked, were placed in Petri dishes on plastic tubes or rods laid across the bottom of the dish. Marked areas on the slide were stained by placing two drops of conjugate (normal-mouse-brain-diluted conjugate to the marked area near the frosted end of the slide and CVS-infected-brain-diluted conjugate, the specificity control, to the other marked area). The conjugate was spread uniformly, without disturbing the film, by means of a toothpick (fresh stick was used for each area stained). Enough water was added to the Petri dish to wet a piece of towel paper laid on the bottom of the dish. After replacing the lid, the Petri dish containing the slides were placed in an incubator at 37°C. After half-an-hour of incubation, the slides were taken out of the incubator and individually washed with a wash bottle of phosphate buffered saline (pH 7.6). The slides were further washed, as a group, in three separate buffered saline (PBS) solutions for a total of 10 minutes. The slides were rinsed, individually, with distilled water. After drying, aided by careful blotting with clean tissue paper, a drop of buffered glycerin was applied to each stained area. Clean cover glass was mounted on each stained marked area. Stained areas were then examined by UV-microscopy.

APPENDIX 5

Rabies Viremia Study by FRAT and MIT

Table 1. Results of fluorescent rabies antibody test (FRAT) and mouse inoculation test (MIT) on blood from CF-1 mice nasally infected with skunk rabies isolate #5072*.

Post-Inoculation Time of Blood Sampling	No. of Mice Bled And Clinical Status When Bled	FRAT	MIT	
			No. of Mice Inoculated	Status & Obsv. Pd.
1 hour	2 mice, a.n.	negative	3 mice	Alive, 82 days
3 hours	2 mice, a.n.	negative	3 mice	Alive, 82 "
6 hours	2 mice, a.n.	negative	3 mice	Alive, 82 "
12 hours	2 mice, a.n.	negative	3 mice	Alive, 83 "
1 day	2 mice, a.n.	negative	3 mice	Alive, 83 "
2 days	2 mice, a.n.	negative	3 mice	Alive, 83 "
3 days	2 mice, a.n.	negative	3 mice	Alive, 79 "
4 days	2 mice, a.n.	negative	3 mice	Alive, 79 "
5 days	2 mice, a.n.	negative	3 mice	Alive, 79 "
6 days	2 mice, a.n.	negative	3 mice	Alive, 74 "
7 days	2 mice, a.n.	negative	3 mice	Alive, 74 "
8 days**	2 mice, a.n.	negative	---	---
9 days	2 mice, a.n.	negative	3 mice	Alive, 70 days
10 days	2 mice, a.n.	negative	3 mice	Alive, 70 "
11 days	2 mice, a.n.	negative	3 mice	Alive, 70 "
12 days	2 mice, a.n.	negative	3 mice	Alive, 66 "
13 days	1 mouse, a.n.; 1 mouse parl	negative	4 mice	Alive, 66 "
14 days	1 mouse, a.n.; 1 mouse, parl.	negative	4 mice	Alive, 66 "
15 days	1 mouse, a.n.; 1 mouse, parl.	negative	4 mice	Alive, 61 "
16 days	1 mouse, a.n.; 1 mouse, parl.	negative	4 mice	Alive, 61 "
17 days	1 mouse, a.n.; 2 mice, parl.	negative	5 mice	Alive, 57 "
18 days	3 mice, parl.	negative	5 mice	Alive, 57 "
19 days	3 mice, parl.	negative	5 mice	Alive, 57 "
20 days	2 mice, parl.	negative	4 mice	Alive, 55 "
21 days	2 mice, parl.	negative	4 mice	Alive, 55 "

*10^{-3} of salivary gland with MLD50 titer of $10^{6.4}$ per 0.03 gm

**MIT not done

Abbreviations used: obsv. pd. - observation period;
a.n. - apparently normal
parl. - paralyzed

Continuation... APPENDIX 5

Table 2. Results of fluorescent rabies antibody test (FRAT) and mouse inoculation test (MIT) on blood from CF-1 mice intramuscularly infected with skunk rabies isolate #5072*.

Post-Inoculation Time of Blood Sampling	No. of Mice Bled And Clinical Status When Bled	FRAT	MIT	
			No. of Mice Inoculated	Status and Obsv. Pd.
1 hour	2 mice, a.n.	negative	3 mice	Alive, 82 days
3 hours	2 mice, a.n.	negative	3 mice	Alive, 82 "
6 hours	2 mice, a.n.	negative	3 mice	Alive, 82 "
12 hours	2 mice, a.n.	negative	3 mice	Alive, 83 "
1 day	2 mice, a.n.	negative	3 mice	Alive, 83 "
2 days	2 mice, a.n.	negative	3 mice	Alive, 83 "
3 days	2 mice, a.n.	negative	3 mice	Alive, 79 "
4 days	2 mice, a.n.	negative	3 mice	Alive, 79 "
5 days	2 mice, a.n.	negative	3 mice	Alive, 79 "
6 days	2 mice, a.n.	negative	3 mice	Alive, 74 "
7 days	2 mice, a.n.	negative	3 mice	Alive, 74 "
8 days**	2 mice, a.n.	negative	---	---
9 days	2 mice, a.n.	negative	3 mice	Alive, 70 days
10 days	2 mice, a.n.	negative	3 mice	Alive, 70 "
11 days	2 mice, a.n.	negative	3 mice	Alive, 70 "
12 days	2 mice, a.n.	negative	3 mice	Alive, 66 "
13 days	2 mice, a.n.	negative	3 mice	Alive, 66 "
14 days	2 mice, a.n.	negative	3 mice	Alive, 66 "
15 days	2 mice, a.n.	negative	3 mice	Alive, 61 "
16 days	2 mice, a.n.	negative	3 mice	Alive, 61 "
17 days	1 mouse, a.n.; 1 mouse, sick	negative	4 mice	Alive, 57 "
18 days	2 mice, a.n.	negative	4 mice	Alive, 57 "
19 days	2 mice, a.n.	negative	4 mice	Alive, 57 "
20 days	1 mouse, a.n.; 1 mouse, sick	negative	4 mice	Alive, 55 "
21 days	1 mouse, a.n.; 2 mice, sick	negative	5 mice	Alive, 55 "
22 days**	2 mice, sick	negative	---	---
23 days**	2 mice, sick	negative	---	---
24 days**	3 mice, sick	negative	---	---
25 days**	2 mice, sick	negative	---	---

* 10^{-3} of salivary gland with MLD50 titer of $10^{6.4}$ per 0.03 gm

**MIT not done

Abbreviations used: obsv. pd. - observation period
a.n. - apparently normal

Continuation ..... APPENDIX 5

Table 3. Results of fluorescent rabies antibody test (FRAT) and mouse inoculation test (MIT) on blood from CF-1 mice orally infected by stomach entubation with skunk isolate #5072*.

Post-Inoculation Time of Blood Sampling	No. of Mice Bled And Clinical Status When Bled	FRAT	MIT	
			No. Mice Inoculated	Status and Obsv. Pd.
1 hour	2 mice, a.n.	negative	3 mice	Alive, 82 days
3 hours	2 mice, a.n.	negative	3 mice	Alive, 82 "
6 hours	2 mice, a.n.	negative	3 mice	Alive, 82 "
12 hours	2 mice, a.n.	negative	3 mice	Alive, 83 "
1 day	2 mice, a.n.	negative	3 mice	Alive, 83 "
2 days	2 mice, a.n.	negative	3 mice	Alive, 83 "
3 days	2 mice, a.n.	negative	3 mice	Alive, 79 "
4 days	2 mice, a.n.	negative	3 mice	Alive, 79 "
5 days	2 mice, a.n.	negative	3 mice	Alive, 79 "
6 days	2 mice, a.n.	negative	3 mice	Alive, 74 "
7 days	2 mice, a.n.	negative	3 mice	Alive, 74 "
8 days**	2 mice, a.n.	negative	---	---
9 days	2 mice, a.n.	negative	3 mice	Alive, 70 days
10 days	2 mice, a.n.	negative	3 mice	Alive, 70 "
11 days	2 mice, a.n.	negative	3 mice	Alive, 70 "
12 days	2 mice, a.n.	negative	3 mice	Alive, 66 "
13 days	2 mice, a.n.	negative	3 mice	Alive, 66 "
14 days**	2 mice, a.n.	negative	---	---
15 days	2 mice, a.n.	negative	3 mice	Alive, 61 days
16 days	2 mice, a.n.	negative	3 mice	Alive, 61 "
17 days	2 mice, a.n.	negative	3 mice	Alive, 57 "
18 days	1 mouse, a.n.; 1 mouse, parl.	negative	4 mice	Alive, 57 "
19 days	1 mouse, a.n. 1 mouse, sick	negative	4 mice	Alive, 57 "
20 days	2 mice, a.n.	negative	4 mice	Alive, 55 "
21 days	2 mice, a.n.	negative	4 mice	Alive, 55 "

* 10^{-3} of salivary gland with MLD50 titer of $10^{6.4}$ per 0.03 gm

**MIT not done

Abbreviations used: obsv. pd. - observation period
a.n. - apparently normal
parl. - paralyzed

APPENDIX 6

Table of fluorescent rabies antibody test (FRAT) results on selected organs of each mouse dead or moribund included in the study of the tissue distribution of rabis virus isolate #5072.

Inoc. Group, Mouse No., and Status at Post-Inoculation Day	FRAT Result						
	Brain	Gastrocnemius	Heart	Kidney	Liver	Lung	Spleen
Intramuscular							
M1, moribund, 18	+	+	-	-	+	+	-
M2, dead, 21	+	-	+	+	-	+	-
M3, dead, 23	+	+	-	+	-	+	+
M4, moribund, 23	+	+	+	+	-	-	-
M5, dead, 23	+	+	+	+	-	+	+
M6, dead, 24	+	+	+	+	+	+	-
M7, dead, 25	+	+	+	+	-	+	-
M8, dead, 25	+	+	+	+	+	+	-
M9, dead, 26	+	+	+	+	+	-	+
M10, moribund, 27	+	+	+	+	+	+	-
Intranasal							
M11, moribund, 14	+	+	-	+	-	+	-
M12, dead, 15	+	+	+	+	-	-	-
M13, dead, 16	+	+	-	+	+	+	+
M14, dead, 18	+	+	+	+	-	+	-
M15, dead, 18	+	+	+	-	+	+	-
M16, moribund, 18	+	-	-	+	+	+	+
M17, dead, 19	+	+	+	+	-	+	+
M18, dead, 19	+	+	+	-	+	+	+
M19, dead, 20	+	+	+	+	-	+	+
M20, dead, 21	+	+	+	+	-	+	-
Oral							
M21, dead, 18	+	+	+	+	-	+	-
M22, dead, 20	+	+	+	+	+	+	+

APPENDIX 7

Table of individual mouse's death time from day of exposure to the skunk rabies virus isolate #5072, according to the route of inoculation.

Route of Inoculation	Individual Time of Rabies Deaths (Post-Inoculation Days)	Group Average Time of Rabies Death
Oral (stomach entubation)	18 and 20	19 days
Nasal	13; 14; 15, 15; 16, 16; 17, 17, 17, 17; 18, 18, 18, 18, 18, 18; 19, 19, 19, 19, 19, 19; 19, 19, 19, 19; 20, 20; 20, 20, 20, 20, 20, 20; 20, 20; 21, 21, 21; 22	18.4 days
Intramuscular	18; 21; 23, 23; 24, 24; 24, 24; 25, 25; 26; 27	23.6 days
Intracerebral*	12, 12; 13, 13; 15	13 days

*From titration study

INFECTIVITY AND DISTRIBUTION OF SKUNK RABIES VIRUS IN MICE FOLLOWING ORAL AND PARENTERAL ADMINISTRATION

by

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D.V.M., University of the Philippines, 1970

AN ABSTRACT OF A MASTER'S THESIS

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ABSTRACT

Rabies is one of the oldest diseases known to mankind. Since the time of Pasteur evidence has accumulated that the rabies virus is disseminated throughout the body through neural pathways. However, some workers (e.g., Dean et al., 1963) believe that bloodborne infection of the brain is possible. Evidence for hematogenous spread of the virus has been presented by Krause (1957,1966), Kitselman and Mital (1967), Shashenko and Kovalev (1971), Gorshunova et al. (1976) and Vanag (1978). Others failed to find rabies virus in the blood (Schindler, 1961; Baer et al., 1965; Murphy et al., 1973). Failure to consistently demonstrate the rabies virus in blood has been the missing evidence to support the bloodborne rabies infection hypotheses.

It is important to reinvestigate the problem of rabies viremia in view of recent reports and because of the tremendous implications consequent to proving its occurrence. Implications, aside from the pathogenesis, would be numerous and could be related to nonbite transmission. Nonbite rabies transmission has been proven, with the oral and nasal openings being shown to be portals of entry of the virus. Infection by ingestion or inhalation may have considerable epidemiologic significance (National Research Council Subcommittee on Rabies, 1973).

This study dealt with rabies viremia in mice infected with an isolate from a naturally rabid skunk. While a bite

is the classic portal of entry for the rabies virus, nonbite transmission can occur. Therefore, the latter was considered in this study of rabies viremia by inoculating experimental animals orally and nasally. This also provided an opportunity to compare the infectivity of the skunk isolate by these routes of inoculation.

Mice were infected by intramuscular, intranasal, or oral administration of a standardized dose of rabies virus in a skunk salivary gland. Blood samples were drawn periodically from these infected groups until rabies mortalities occurred. The brain, liver, lung, kidney, heart, spleen, blood, and gastrocnemius muscle of dead or dying mice were sampled to determine the tissue distribution of the virus. The fluorescent rabies antibody test (FRAT) was applied to all tissues. The standard mouse inoculation test (MIT) was also used to determine the presence of a viremia, by intracerebrally inoculating blood samples into weanling mice.

Viremia was not proved in this study. Neither FRAT nor MIT gave positive results even though 92 per cent of the nasally inoculated and 28 per cent of the intramuscularly injected mice died from the rabies infection. Mice inoculated by stomach entubation were the least susceptible to the virus; only 5 per cent died in spite of receiving a dose ten times higher than those of the other groups. While the virus was not detected in the blood of any mice, most of the parenchymal tissues were positive by the FRAT. Such a generalized viral distribution--important in rabies epidemiology especially nonbite transmission--was thought to be due to

the centrifugal spread of the virus from the central nervous system. The brain was positive in all of these mice and the disease process was terminal.