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EXPERIMENTAL INOCULATION OF RACCOONS (*PROCYON LOTOR*) WITH RABIES VIRUS OF SKUNK ORIGIN

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ABSTRACT: To determine raccoon (*Procyon lotor*) susceptibility and serum neutralizing antibody response to a skunk salivary gland rabies virus, raccoons were inoculated with a rabies virus isolated from a naturally-infected striped skunk (*Mephitis mephitis*). Raccoons were divided into four groups of three animals each. A dilution of the rabies virus suspension, $10^{2.4}$, $10^{3.4}$, or $10^{4.4}$, mouse intracerebral lethal dose$_{50}$ (MICLD$_{50}$), was administered into the masseter muscles of each animal. Three negative control animals received only diluent. Saliva and sera were collected on post-inoculation days 35, 63 and 92 for virus isolation and determination of serum neutralizing antibody titer. All animals survived the 92 day observation period and none exhibited the behavioral changes classically associated with clinical rabies virus infections. Rabies virus was not detected in the saliva of any raccoon and two of the three animals receiving the highest inoculum developed serum neutralizing antibodies (SNA). On day 92, a challenge suspension of New York City/Georgia (NYC/GA) strain rabies virus in fox salivary glands ($10^{3.2}$ MICLD$_{50}$) was administered to all 12 raccoons. All animals succumbed to rabies virus except the two animals that had earlier developed SNA. The results of this study provided evidence about the susceptibility of raccoons to a skunk rabies virus and demonstrated that exposed raccoons could survive for at least 92 days following exposure. Furthermore, animals developing SNA under such circumstances were capable of withstanding challenge with rabies virus that was fatal for seronegative raccoons.

Key words: Rabies, skunk origin rabies virus, raccoons, *Procyon lotor*, inoculation, experimental study.

INTRODUCTION

Experiments designed to study raccoon susceptibility to wildlife rabies viruses have shown that raccoons are less susceptible to certain isolates of rabies virus than some other animal species. Raccoons were found to be 1,000 times more resistant than foxes to a challenge with a fox salivary gland isolate (Sikes and Tierkel, 1961). Using a fox isolate, a $10^{2.2}$ mouse lethal dose$_{50}$ (MLD$_{50}$) produced 50% mortality and even $10^{4.2}$ MLD$_{50}$ produced only 72% mortality in raccoons. In contrast, 24 of 26 foxes died when inoculated with the same virus at doses ranging from $10^{1.1}$ to $10^{4.1}$ MLD$_{50}$. In a separate study, the fatal intramuscular dose in raccoons was found to be 10^3 mouse intracerebral lethal dose$_{50}$ (MICLD$_{50}$) of a red fox virus strain (Artois et al., 1989). In other studies, raccoons remained clinically normal for 104 days (day of euthanasia) following challenge with $10^{3.4}$ MLD$_{50}$ of a Mexican free-tailed bat virus (Constantine, 1966a), and for 243 days (day of euthanasia) following bites by rabid red bats (Constantine and Woodall, 1966). These data suggested that raccoons are less susceptible to certain strains of rabies virus.

In the central United States, enzootic skunk rabies predominates (Charlton et al., 1987). Rabies surveillance data from the state of Iowa illustrate this occurrence (Iowa Department of Public Health 1981–90). During the 1980's, there were 1,887 confirmed cases of rabies in the striped skunk. In contrast, there were only 14 confirmed cases in raccoons. Similarity in range distributions and overlapping ecological niches of raccoons and skunks should allow for exposure of raccoons to rabid skunks, but susceptibility of raccoons to the skunk virus is unknown. The objectives of this study were to assess the susceptibility of raccoons to a skunk rabies isolate and to monitor the serum neutralizing (SN) antibody response in raccoons exposed to increasing quantities of skunk rabies virus under experimental conditions.

MATERIALS AND METHODS

Virus inoculum and titration

The rabies virus suspension consisted of a homogenate of salivary glands from a naturally infected striped skunk submitted to the Iowa Veterinary Diagnostic Laboratory (Ames, Iowa 50011, USA). Mandibular salivary glands were ground with mortar and pestle in 30 ml of diluent consisting of 2% normal horse serum in water, pH 7.6, containing 500 units of penicillin and one mg of streptomycin per ml. The suspension was stored at −70 C until use.

The virus suspension was titrated by intracerebral inoculation in mice (Koprowski, 1973). Groups of ten 13–15 gram female white mice (Sprague Dawley CF-1 mice, Harlan Sprague Dawley, Inc., Indianapolis, Indiana 46229, USA) were inoculated with 0.03 ml of each virus dilution and observed for 21 days. 50% mortality endpoint calculations were made using the Reed-Muench formula (Reed and Muench, 1938).

Animals

Twelve adult farm-reared raccoons (Ruby's Fur Farm, New Sharon, Iowa 50207, USA) were used in the experiment. The smallest number of animals possible was selected for this study without compromising significance of the results. The animals were housed individually in stainless steel cages in an isolation facility in accordance with USDA animal care standards. The experimental protocol was approved by the Iowa State University Animal Welfare Committee. Upon arrival, all animals were treated for parasites, identified by ear tags, and weighed. Each animal was tested for rabies serum neutralizing antibodies (SNA) 14 days prior to, and on the day of, inoculation. Animals were randomly divided into four treatment groups of three animals each.

Inoculation procedure and sample collection

Animals were anesthetized with a mixture of 2 mg/kg ketamine hydrochloride (Ketaset, Bristol Laboratories, Syracuse, New York 13220, USA), 2 mg/kg xylazine (Rompun, Haver-Lockhart Laboratories, Shawnee, Kansas 66203, USA), and 0.2 mg/kg butorphanol tartrate (Torbugesic, Bristol Laboratories, a division of Bristol-Myers Co., Syracuse, New York 13221, USA) administered intramuscularly. The rabies inoculum consisted of 1 ml of the skunk salivary gland suspension diluted to virus titer of $10^{2.4}$, $10^{3.4}$, or $10^{4.5}$ mouse intracerebral lethal $dose_{50}$. For each dilution, three raccoons were inoculated with 0.5 ml of the virus suspension in each masseter muscle. Three animals received only diluent. Animals were observed daily for changes in behavior. Animals were anesthetized on days 35, 63 and 92, and weighed. Blood samples for monitoring antibody response were collected by jugular vein or cardiac puncture. Saliva samples for virus isolation were collected by swabbing the oral mucosa and tonsilar areas with sterile cotton swabs.

On day 92, a suspension of New York City/Georgia (NYC/GA) canine strain virus (Rabies virus, National Veterinary Services Laboratories, Ames, Iowa 50013, USA) in fox salivary glands ($10^{3.2}$ $MICLD_{50}$) was administered to all raccoons in the masseter muscles. The NYC/GA virus challenge level for the 12 animals in this study was determined from an initial titration study in six raccoons in which a raccoon lethal $dose_{50}$ of $10^{1.8}$ $MICLD_{50}$ of the NYC/GA virus was determined (R. E. Hill, unpubl. data). Animals were observed daily for changes in behavior. Sera were collected at the time of death or at 29, 61 and 90 days following NYC/GA challenge. Rabies deaths were confirmed by fluorescent antibody (Fluorescein isothiocyanate conjugated equine or bovine origin antirabies globulin, BBL Microbiology systems, Becton Dickinson and Co., Cockeysville, Maryland 21030, USA) staining of acetone-fixed impression smears of brain tissue (Dean and Abelseth, 1973). Surviving raccoons were sacrificed on day 90 following NYC/GA rabies virus challenge and examined for evidence of rabies virus infection. Samples were collected for fluorescent antibody staining, mouse inoculation (Koprowski, 1973), and histologic examination.

Serum neutralizing antibody titer determination

Sera were analyzed in triplicate by the rapid fluorescent focus inhibition test (RFFIT) for the presence of antibodies against rabies virus (Smith et al., 1973). Serial five-fold dilutions were made and the titer expressed as the geometric mean of the reciprocal of the highest dilution capable of reducing the number of rabies infected cells by 50% as determined by the Reed and Muench method (Reed and Muench, 1938). Sera were tested in baby hamster kidney (BHK-21(C-13)) cells (Cells originated from the Centers for Disease Control and were supplied by the National Veterinary Services Laboratories, Ames, Iowa 50013, USA) using Challenge Virus Standard (CVS-11) virus (Rabies virus, National Veterinary Services Laboratories, Ames, Iowa 50013, USA). Test controls for the RFFIT included uninfected cell controls, back titration of rabies virus challenge dose, negative antirabies sera and titration of positive raccoon antirabies sera from raccoons previously vaccinated with a killed vaccine (Rabguard-TC, Norden Laboratories, Lincoln, Nebraska 68501, USA).

TABLE 1. Rabies virus-neutralizing antibody titers and response to inoculation with two rabies virus isolates in raccoons.

Group	Raccoon number	Inoculum[a] $MICLD_{50}$	RFFIT[b] antibody titer DPI 35	DPI 63	DPI 92	Challenge[c] $MICLD_{50}$	Response to challenge[d]	RFFIT[e] antibody titer Day of death	DPC 29	DPC 61	DPC 90
A	5		<4.0	<4.0	<4.0		D (15)	<4.0			
	11	235 ($10^{2.4}$)	<4.0	<4.0	<4.0	1,585 ($10^{3.2}$)	D (12)	<4.0			
	12		<4.0	<4.0	<4.0		D (17)	<4.0			
B	1		<4.0	<4.0	<4.0		D (21)	31.5			
	3	2,400 ($10^{3.4}$)	<4.0	<4.0	<4.0	1,585 ($10^{3.2}$)	D (18)	<4.0			
	10		<4.0	<4.0	<4.0		D (21)	<4.0			
C	2[f]		7.1	4.1	<4.0		S		9.4	<4.0	<4.0
	7	63,200 ($10^{4.8}$)	<4.0	<4.0	<4.0	1,585 ($10^{3.2}$)	D (15)	32.0			
	9		27.6	29.4	24.3		S		35.9	10.3	<4.0
D	4		<4.0	<4.0	<4.0		D (14)	8.6			
	6	Controls	<4.0	<4.0	<4.0	1,585 ($10^{3.2}$)	D (15)	<4.0			
	8		<4.0	<4.0	<4.0		D (15)	5.5			

[a] Groups were inoculated with 1 ml of a skunk rabies virus suspension (0.5 ml in each masseter muscle). Controls received 1 ml diluent. $MICLD_{50}$ = Mouse intracerebral lethal $dose_{50}$.

[b] Virus-neutralizing antibody titer as determined by the rapid fluorescent focus inhibition test (RFFIT) at specified number of days post skunk virus inoculation (DPI). A titer of <4.0 is considered negative.

[c] All animals were challenged on DPI 92 with 1 ml ($10^{3.2}$ $MICLD_{50}$) of New York City/Georgia (NYC/GA) rabies virus (0.5 ml in each masseter muscle).

[d] S = Survived, D = Died. Day of death following NYC/GA challenge is shown in parentheses.

[e] Virus-neutralizing antibody titer on day of death or at specified number of days post NYC/GA rabies virus challenge (DPC).

[f] This raccoon developed mild peripheral neurologic signs evident since day 13 following initial inoculation.

Virus isolation

Saliva swabs were suspended in 1 ml of Glasgow minimum essential medium (Gibco Laboratories, Grand Island, New York 14072, USA) and placed in an ice bath immediately following collection. Samples were stored frozen (−70 C) until tested. Each sample was tested for the presence of rabies virus in murine neuroblastoma cells (Cells originated from the Centers for Disease Control and were supplied by the National Veterinary Services Laboratories, Ames, Iowa 50013, USA) (Webster and Casey, 1988). Skunk and NYC/GA virus infected cells were used as positive controls.

RESULTS

Skunk virus inoculation

None of the animals developed fatal rabies. Raccoon #2 developed a neurologically-related clinical sign: a tic in the left front leg on day 13 that slowly progressed into a bilateral twitch of the front legs and shoulders which was seen when the animal was in a sitting position. It is uncertain if rabies virus was involved with the development of this sign. In all other aspects, this animal behaved normally. No adverse reactions were observed in any of the other 11 raccoons. Virus was not detected in any of the saliva samples. All 12 animals continued to gain weight throughout the 92 day observation period. Two of the nine virus inoculated animals had developed SNA by day 35 including raccoon #2. These two animals again tested positive on day 63, but only one animal had detectable SNA by day 92. Table 1 gives the SN antibody levels for each raccoon.

NYC/GA virus challenge

Two of the nine animals previously inoculated with the skunk virus also survived the challenge with NYC/GA rabies virus. All controls (three of three) died within 15 days following challenge. Table 1 gives the post-challenge results for each raccoon. Challenged raccoons first showed clinical signs from 11 to 21 days after exposure. Three raccoons were found dead in their cages without clinical signs. Of those rac-

coons showing clinical signs there were variations, but most animals followed a pattern. Animals had a short period of increased alertness or apprehension (<24 hr), followed by extremely aggressive behavior. All animals showing aggressive behaviors died within a few hours of the onset of these signs. Several animals were observed to drink during this phase.

None of the seven animals which had not developed SNA following the skunk virus inoculation survived the NYC/GA virus challenge. One of these seven raccoons seroconverted following the NYC/GA virus challenge, as did two of the three controls.

Both animals that had previously developed SNA survived the NYC/GA virus challenge and both had increased SN antibody titers on day 29 following the NYC/GA challenge. No evidence of viral infection was observed by fluorescent antibody staining, or mouse inoculation of brain tissue of these two animals at necropsy. No evidence of viral infection was observed by histologic examination of the nerves of brachial plexus and corresponding dorsal root ganglia in the raccoon which exhibited the left front leg tic.

DISCUSSION

In regions of the United States with wildlife rabies, clinically normal free-ranging raccoons have been found with SNA against rabies virus. Seropositive frequency rates ranged from 0 to 5.6% in areas free of enzootic raccoon rabies (McLean, 1975) and from three to 28% in areas with enzootic raccoon rabies (Bigler et al., 1983; Jenkins et al., 1988). These findings initially raised the question of survivability of raccoons exposed to rabies virus in the field.

There are a limited number of reports of the development and protective nature of SNA in raccoons. Raccoons bitten by rabid red bats have been shown to develop SNA while remaining clinically normal (Constantine and Woodall, 1966). Similar results were reported in raccoons inoculated with a salivary gland suspension from coyotes previously infected with Mexican free-tailed bat virus (Constantine, 1966a, b).

Many factors are at work in survivability and transmission of rabies in different species. The geographically restricted, compartmentalized, single reservoir association of rabies virus has been described (Smith et al., 1986). In addition, each animal species seems to have an inherent level of susceptibility to any rabies virus (World Health Organization, 1973). Individual animal responses to virus challenge complicate the pathogenesis even further.

It is also recognized that each species adapted virus behaves differently in alternate hosts. The variability of survival among wildlife species following rabies challenge with different virus variants is well documented (Sikes, 1962; Parker and Wilsnack, 1966; Sikes and Tierkel, 1961). For example, previous experimental raccoon exposure studies have shown that they are less susceptible to experimental infection with fox virus (Sikes and Tierkel, 1961). Death in 100% of the animals was not seen in any dilution up to $10^{4.2}$ MLD_{50}. Other factors, such as route of exposure and method of challenge preparation are known to affect pathogenicity (Soulebot et al., 1982).

This study demonstrated that raccoons are less susceptible to intramuscular inoculation with even high levels of skunk virus. Although the raccoons in this study were given a higher dose of skunk virus than was previously shown to be lethal in both skunks and foxes (Parker and Wilsnack, 1966), they survived for 92 days following exposure. The development of SNA in raccoons exposed to skunk virus appeared to be related to the dose of inoculum.

Serologic surveys in both raccoon rabies enzootic areas and areas with other species as primary vectors have found that wild-trapped raccoons have SNA. This study demonstrated that experimentally inocu-

lated animals can develop SNA and survive for at least 92 days. In this case 22% (2/9) of the animals receiving the skunk virus developed rabies-specific SNA. These two animals were both from a group of three raccoons that had received $10^{4.8}$ $MICLD_{50}$ of the virus. Development of SNA in raccoons appeared to be related to dose of virus received. Similar results have been reported in skunks (Charlton et al., 1987).

Virus was not detected in any of the saliva samples. This is not unexpected because in other species, shedding of virus for more than a few days prior to the onset of clinical signs has not been reported and the route to the salivary glands is via the central nervous system (Schneider, 1975).

Because of the similarity of the range distributions and overlapping ecological niches of striped skunks and raccoons in the United States, occasional exposure of raccoons to rabid skunks is likely. Although there is considerable variation in the amount of virus rabid skunks excrete in their saliva, large amounts of virus can be excreted (Sikes, 1962; Parker and Wilsnack, 1966; Vaughn, 1963, 1965). With all of the animals in this study surviving the inoculation with skunk virus, it appeared that inapparent infections or long incubation periods in exposed raccoons may be possible. It remains unknown if any of the animals inoculated with the skunk virus would have come down with clinical rabies after more than 92 days.

The results of the NYC/GA virus inoculation study indicated that animals previously exposed to the skunk virus could survive lethal challenge. Both animals that had developed SNA survived the NYC/GA virus challenge, including the animal whose titer ultimately dropped below 4.0. Both of these animals had an anamnestic increase in SN antibody titer by day 29 following NYC/GA challenge. In general, correlation between protection from challenge and SN antibody titer has been positive (MacFarlan, 1988), yet in any host, absolute correlation of protection from lethal challenge and SN antibody levels has been lacking. This study supported the hypothesis that SNA was a primary factor involved in protection from lethal challenge. In addition, there appeared to be some degree of immune system memory involved in protection as evidenced by the survival of the one raccoon whose titer dropped below 4.0.

The incubation period of the skunk virus in raccoons and the possibility of inapparent infection in exposed raccoons needs further investigation. It needs to be determined if exposed raccoons represent a source of secondary transmission to people and other animals, and if the distribution of the virus in exposed raccoons is such that it ever reaches the salivary glands. The mechanism of raccoon survival following experimental inoculation needs to be explored.

The overall picture of raccoon susceptibility to a skunk adapted rabies virus is still uncertain. More work in larger numbers of raccoons with different challenge levels and longer observation periods is needed before definitive conclusions can be drawn. Continued virus isolations and monoclonal antibody characterizations of rabies viruses isolated from raccoons found rabid in skunk endemic rabies areas could further delineate if the viruses are distinct variants from skunk, bat, or other sources (Smith et al., 1986). In addition, experimental studies in skunks with a raccoon adapted virus are needed as the host-associated enzootic areas of these two species begin to overlap.

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