Experimental Rabies in Skunks: Mechanisms of Infection of the Salivary Glands

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ABSTRACT

Striped skunks (*Mephitis mephitis*) were inoculated into the right submandibular salivary gland with street rabies virus. They were killed at various times after inoculation and several tissues were examined by immunofluorescence and light microscopy. Right and left superior cervical, nodose and trigeminal ganglia, medulla oblongata and at least three regions of right and left submandibular salivary glands were examined by the fluorescent antibody technique. Intracerebral titrations of salivary gland suspensions were made in weanling white Swiss mice.

Immunofluorescent material (inoculum) was detected in septa and connective tissue surrounding secretory units of the right submandibular gland immediately after inoculation, but otherwise antigen was not detected in either right or left submandibular glands without coincident antigen in the medulla oblongata. This occurred first on day 12 in areas of the gland remote from the inoculation site. Titers of virus were low at this time. Serum neutralizing antibodies occurred by day 7 in a few skunks. The time of development and distribution of antigen strongly suggest that, even after direct inoculation, neural networks are necessary for development of widespread infection of the salivary gland. The early occurrence of serum neutralizing antibodies in some of the skunks suggests that the immune response was activated by virus in the inoculum since immunofluorescence was not detected in any tissue at this time.

Key words: Rabies, skunks, salivary gland infection, centrifugal movement of virus.

RÉSUMÉ

Cette expérience consistait à inoculer le virus de la rage des rues dans la glande salivaire sous-maxillaire droite de mouffettes rayées (*Mephitis mephitis*). On les sacrifia ensuite à différents intervalles et on examina plusieurs de leurs tissus par l'immunofluoresence et la microscopie photonique. On soumit à l'examen, par la technique d'immunofluorescence, les ganglions cervicaux supérieurs et noueux droits et gauches, ainsi que ceux des nerfs trijumeaux, le bulbe rachidien et au moins trois sites de la glande salivaire sous-maxillaire droite et de la gauche. On utilisa des souris blanches récemment sevrées pour effectuer les titrations intra-cérébrales des suspensions des glandes salivaires.

Immédiatement après l'inoculation, le tissu conjonctif entourant les lobules et les acini de la glande salivaire sous-maxillaire droite recelait des particules fluorescentes qui correspondaient à l'inoculum; plus tard, on ne décela pas d'antigène rabique dans cette glande ou dans celle du côté gauche, sans en déceler aussi dans la moelle allongée. Cette constatation se produisit d'abord 12 jours après l'inoculation, dans des régions de la glande éloignées du site d'inoculation, et la concentration de virus s'y révéla plutôt faible. Au bout de sept jours, des anticorps sériques neutralisants firent leur apparition, chez quelques mouffettes. Le temps requis pour le développement et la distribution de l'antigène rabique suggèrent fortement la nécessité, même après une inoculation directe, d'un lacis de filets nerveux pour permettre le développement d'une infection diffuse de la glande salivaire. L'apparition hâtive d'anticorps sériques neutralisants, chez certaines mouffettes, laisse supposer que le virus de l'inoculum stimula la réaction immunologique, puisqu'aucun tissu n'affichait alors d'immunofluorescence.

Mots clefs: rage, mouffettes, infection des glandes salivaires, mouvement centrifuge du virus rabique.

INTRODUCTION

The transmission of rabies in nature is usually due to transfer of infective oral fluids by biting rabid animals. The sources of virus for these fluids include the salivary

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glands (extrinsic and intrinsic) and other oral and nasal tissues (nasal glands and oral and nasal epithelium) (1,2,3,4,5,6,7,8,9). A recent study of naturally infected skunks indicated that the concentration of virus generally was highest in the mandibular glands, moderate in the parotid glands and low in the sublingual glands (10).

Previous studies indicate that infection of the central nervous system (CNS) precedes salivary gland infection (9,11), that infection occurs via peripheral nerves (12,13,14), and that glandular epithelial cells support growth and release of virus (1,4,5,6,9,15,16, 17,18). Titers of virus in salivary glands may exceed those in the brain (15,16,17).

Although it is well established that the route of infection from the CNS to the salivary glands is via peripheral nerves, the extent of nonneuronal spread of virus within salivary gland tissue has not been reported. This is a study of the development of infection of the skunk submandibular (submaxillary, mandibular) salivary glands following direct inoculation of street virus into one gland.

MATERIALS AND METHODS

VIRUS

A 10% suspension (10^{-1} dilution) of submandibular salivary glands from naturally infected skunks was prepared by homogenization with diluent (0.01 M phosphate buffer, pH 7.4, containing 10% fetal bovine serum, 1000 IU penicillin and 2 mg streptomycin/mL) and centrifugation at 600 g for 15 min. When titrated in weanling mice, the $10^{-6.1}$ dilution contained one mouse intracerebral lethal $dose_{50}$ ($MICLD_{50}$)/0.03 mL.

EXPERIMENTAL ANIMALS

Male and female striped skunks (*Mephitis mephitis*), reared in captivity were purchased from a supplier.[1] They were kept individually in stainless steel cages and given food and water *ad lib*. The skunks were four to six months old at the beginning of the exeriment.

Female weanling white Swiss mice were used for titration of inoculum and for titration of salivary gland suspension and saliva of the experimental skunks.

EXPERIMENTAL PROCEDURE

Experiment 1 — Twenty-eight principals were divided into seven groups of four, each group containing equal numbers of males and females (Table I). Each skunk was anesthetized with ketamine hydrochloride[2] (10 mg/lb) and acepromazine maleate[3] (0.25 mg/lb) given intramuscularly. The right submandibular salivary gland was exposed surgically and, using a 27 gauge 0.5 inch needle, 0.1 mL of 10^{-1} dilution of virus suspension was inoculated into the posteromedial pole of the gland. The site was marked by a stainless steel suture in the capsule of the gland. Four control skunks were inoculated with the same amount of salivary gland suspension from normal skunks.

Blood for serum neutralization tests (19) was collected before inoculation, terminally from all skunks, and at the times listed in Table III. Saliva was collected from all skunks before inoculation, and every 3-4 days until death, by swabbing the mucous membranes of the mouth. The swab was immersed in 2 mL of diluent and the resulting suspension used for intracerebral inoculation of weanling mice.

Groups of four skunks were killed at 1 and 4 h, at 2,4,7 and 12 days and after clinical signs had developed (Table I). Two controls were killed on day 2 and two on day 12.

At necropsy, each submandibular gland was divided into a posterolateral half and an anteromedial half, the line of bisection passing through the point of insertion of the stainless steel suture. A small piece of the posterolateral half was fixed in 10% neutral buffered formalin and the remainder was frozen and used for intracerebral titration in weanling mice. The anteromedial half was divided transversely into four blocks, mounted on pieces of cardboard and frozen in liquid nitrogen. The face of one block was at the inoculation site; the cutting surfaces of the other blocks were approximately equidistant from each other and from the ends of the gland. Other tissues frozen in liquid nitrogen were: medulla oblongata, pons, and right and left trigeminal, nodose and superior cervical ganglia. All the tissues frozen in liquid nitrogen were sectioned at 8 μm and stained by the fluorescent antibody technique using a hamster conjugate (20). Pieces of brain and several visceral organs were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 6 μm and stained with hematoxylin and eosin.

Experiment 2 — Eight skunks were inoculated into the right submandibular salivary gland with 0.1 mL of 10^{-3} dilution of virus suspension (Table II). Two were killed on day 20, two on day 25 and four were killed after clinical signs had developed. Otherwise the procedures were the same as those for experiment 1.

RESULTS

EXPERIMENT 1

In skunks killed at 1 and 4 h, granular immunofluorescent material (inoculum) occurred in thick or thin bands in septa and connective tissue surrounding acini near the inoculation site of the submandibular salivary gland (Table I).

Subsequently no immunofluorescence was detected in any tissue until day 12. Three of four skunks killed on day 12 had immunofluorescence in the brain and in several of the ganglia examined (Table I). Two of these skunks had slight infection of the right submandibu-

[1]Ruby's Fur Farm, New Sharon, Iowa.
[2]Ketaset, Rogar/STB, Mississauga, Ontario.
[3]Atravet, Ayerst Laboratories, Montreal, Quebec.

TABLE I. Experiment 1. Infection of Salivary Glands Following Direct Inoculation of Virus

Time post-inoculation	Skunk No.	Virus Titer[a]		Immunofluorescence[b]				
		Submandibular Salivary Glands		Submandibular Salivary Glands		Selected Ganglia[c]		Medulla Oblongata
		Right	Left	Right	Left	Right	Left	
Hour 1	1	2.5	—	++[d]	—	—	—	—
	2	2.5	—	++[d]	—	—	—	—
	3	2.3	—	++[d]	—	—	—	—
	4	1.5	—	++[d]	—	—	—	—
Hour 4	5	2.7	—	++[d]	—	—	—	—
	6	2.3	—	++[d]	—	—	—	—
	7	3.3	—	++[d]	—	—	—	—
	8	3.1	—	++[d]	—	—	—	—
Day 2	9	—	—	—	—	—	—	—
	10	—	—	—	—	—	—	—
	11	—	—	—	—	—	—	—
	12	—	—	—	—	—	—	—
Day 4	13	—	—	—	—	—	—	—
	14	—	—	—	—	—	—	—
	15	—	—	—	—	—	—	—
	16	—	—	—	—	—	—	—
Day 7	17	—	—	—	—	—	—	—
	18	—	—	—	—	—	—	—
	19	—	—	—	—	—	—	—
	20	—	—	—	—	—	—	—
Day 12	21	—	—	—	—	—	—	—
	22	—	—	—	—	++(T,N,SC)	+(T,N,SC)	+++
	23	1.7	—	+[e]	—	+ (T,SM)	—	++
	24	2.3	—	±[e]	—	+ (T,SM)	+(T)	+
Day 18	25[f]	2.9	4.1	+[e]	+[e]	++(T,N,SC,SM)	++(T,N,SC,SM)	+++
Day 20	26[f]	5.7	5.7	++	++	++(T,N,SC,SM)	++(T,N,SC,SM)	+++
Day 20	27[f]	—	—	—[g]	—[g]	++(T,N)+(SM)	++(T,N)	+++
Day 85	28	—	—	—	—	—	—	—

[a]Log_{10} $MICLD_{50}$/0.03 mL, —, no deaths at 10^{-1} dilution
[b]—, no immunofluorescence detected; ±, very slight, +, slight; ++ moderate; +++, marked; NE, not examined
[c]Right and left trigeminal (T), nodose (N), superior cervical (SC) and submandibular (SM) ganglia
[d]Inoculum in septa and peracinar connective tissue near site of inoculation
[e]Glands with regional distribution
[f]Skunks 25-27 had clinical signs of rabies
[g]In nerve fibers only

TABLE II. Experiment 2. Infection of Salivary Glands Following Direct Inoculation of Virus

Time post-inoculation	Skunk No.	Virus Titer[a]		Immunofluorescence[b]				
		Submandibular Salivary Glands		Submandibular Salivary Glands		Selected Ganglia[c]		Medulla Oblongata
		Right	Left	Right	Left	Right	Left	
Day 20	29	—	—	—	—	—	—	—
	30	—	—	—	—	++(SC)	±(SC)	±
Day 25	31	3.7	1.3	±	±	++(T,N,SM)	++(T,N,SM)	+++
	32	—	—	—	—	+++(T) +(N,SC,SM)	+(T) +(N,SE,SM)	+++
Terminal								
Day 21	33[d]	5.7	4.9	++	++	+++(T,N,SC,SM)	+++(T,N,SC,SM)	+++
Day 26	34[d]	2.7	2.7	++	++	+++(T,N,SC,SM)	+++(T,N,SC,SM)	+++
Day 31	35[d]	3.1	2.7	++	++	+++(T,N,SC) +(SM)	+++(T,N,SC) +(SM)	+++
Day 53	36[d]	<1	1.1	+	++	+++(T,N) ++(SC,SM)	+++(T,N) ++(SC,SM)	+++

[a]Log_{10} $MICLD_{50}$/0.03 mL
[b]Same as Table I
[c]Same as Table I
[d]Skunks 33-36 had clinical signs of rabies

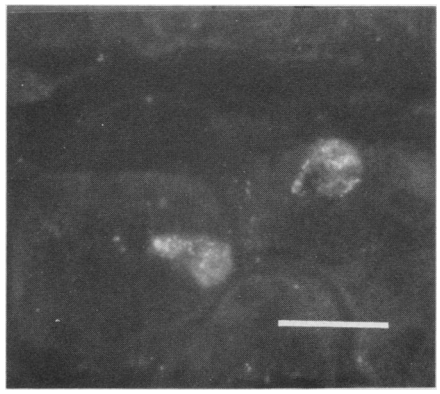

Fig. 1. Two acini. Each one has an epithelial cell containing antigen. Bar indicates 25 μm.

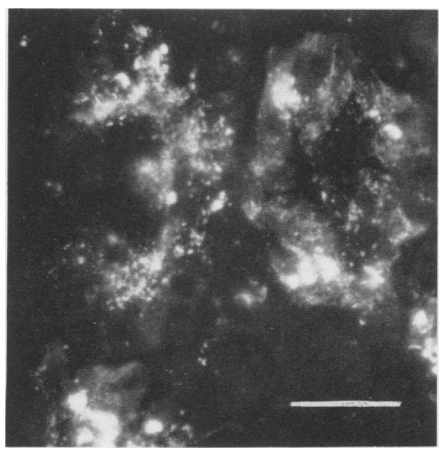

Fig. 2. Extensive immunofluorescence in two adjacent acini. Bar indicates 25 μm.

lar gland, as demonstrated by immunofluorescence. In both cases, the affected region was remote from the inoculation site. Affected acini had one or more antigen-containing epithelial cells (Figs. 1 and 2). Large and small granules of immunofluorescent material occurred throughout the cytoplasm of affected cells, but usually were more numerous in the apical region. Irregular streams of immunofluorescent material were interpreted as aggregations of antigen in acinar lumena and canaliculi. Neurons of the skunk submandibular ganglion occur in small groups in the glandular septa, and many of these contained antigen (Fig. 3, Table I). Linear arrays of immunofluorescent granules occurred in nerve fibers of small nerves within the glands.

Three skunks were killed after

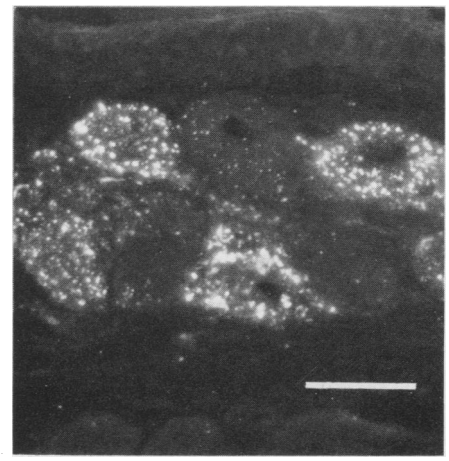

Fig. 3. Neurons of submandibular ganglion containing immunofluorescent granules. Neurons of the submandibular ganglion in the skunk occur singly and in small groups scattered through the septa. Bar indicates 25 μm.

development of clinical rabies (Table I). All three had immunofluorescence in the brain and several ganglia and two had immunofluorescence in glandular epithelium of both right and left submandibular glands. Skunk 27 had antigen in a few nerve fibers and neurons of the submandibular ganglion but none in the glandular epithelium (Table I). One skunk (No. 28) did not develop clinical signs and was killed on day 85. Antigen was not found in any tissue.

With a few exceptions, there was fairly close correlation between grades of immunofluorescence in salivary glands and titers of salivary gland suspension in mice in both experiments 1 and 2 (Tables I and II). By mouse inoculation, virus was detected in saliva of one skunk (No. 26) on day 20; all other saliva samples from this and the other skunks were negative. Four of ten skunks developed SN titers by day 7, and five of eight skunks had titers by day 12. The titers on days 7 and 12 were fairly low whereas two of three skunks that developed clinical signs had high titers (Table III).

Histologically, a few of the skunks killed within a few days of inoculation had moderate accumulations of neutrophils and macrophages in the periglandular connective tissue and septa at the inoculation site. Skunks killed after development of clinical signs had mild sialoadenitis of both right and left submandibular glands. There were regional accumulations of mononuclear cells in septa and a few scattered acini contained necrotic epithelial cells. Necrosis of epithelial cells did not involve all cells in affected acini. Some glands (inoculated and uninoculated of control skunks) had one or a few accumulations of mononuclear cells. Light microscopic lesions in the brain were similar to those previously described (21).

TABLE III. Experiment 1. Serum Neutralization Titers of Skunks Exposed by Direct Inoculation of the Submandibular Salivary Glands

	Postinoculation sample							
	1		2		3		4	
Skunk No.[a]	Time	Titer[b]	Time	Titer	Time	Titer	Time	Titer
17	Day 7	—						
18	Day 7	—						
19	Day 7	0.13						
20	Day 7	0.13						
21	Day 7	—	Day 12	—				
22	Day 7	0.15	Day 12	0.85				
23	Day 7	ND[c]	Day 12	—				
24	Day 7	ND[c]	Day 12	0.39				
25	Day 7	—	Day 12	0.15	Day 18	6.2		
26	Day 7	—	Day 12	0.15	Day 20	0.24		
27	Day 7	—	Day 12	0	Day 20	10.2		
28	Day 7	0.15	Day 12	0.41	Day 74	0.37	Day 85	0.19

[a]Sera from skunks 1-16 taken Hour 1, Hour 2, Day 2 or Day 4 were negative for rabies neutralizing antibodies

[b]Titer in international units. —, <0.13 I.U.

[c]Not done

EXPERIMENT 2

Immunofluorescence was not detected in any tissue of skunk 29 killed on day 20, but was detected in various tissues of all the remaining skunks in this experiment (Table II). Skunk 30 had fairly extensive immunofluorescence in the right superior cervical ganglion, but antigen was detected in only two neurons of the left superior cervical ganglion. There was very slight immunofluorescence in a few neurons in the rostral medulla oblongata but none in any other tissue examined (Table II). Skunk 31 had immunofluorescence in all tissues examined except the superior cervical ganglia.

All skunks killed after development of clinical signs had immunofluorescence in the brain and in all the ganglia examined. None of the skunks had serum neutralizing antibodies before clinical signs began (Table IV). By intracerebral mouse inoculation, saliva from all skunks was negative on days 7 and 12; saliva from skunk 33 was positive on days 19 and 21, saliva from skunk 34 was positive on day 19 and negative on day 26; saliva from skunk 35 was negative on day 19, positive on day 26 and negative on day 31; and skunk 36 had saliva negative on days 19,26,35,41, positive on day 47 and negative on day 53.

The light microscopic lesions were similar to those in skunks of experiment 1.

DISCUSSION

Centrifugal migration of rabies virus from the CNS may result in infection of several nonnervous tissues (9). Our previous studies in skunks suggest that centrifugal infection of skeletal muscle cells is sporadic — involving scattered myocytes in several muscles (21). In naturally occurring rabies in skunks, the submandibular salivary glands frequently contain virus at high titer and antigen may be widespread in glandular epithelium. We have not found any reports to indicate whether this widespread infection of glandular tissue is due to intrinsic cell-to-cell spread among epithelial cells after initial focal infection of the gland or to widespread axonal release of virions.

Our results suggest that, even after direct inoculation, neural networks are essential to achieve widespread infection of glandular tissue. After direct inoculation, antigen was not detected in epithelial cells without concurrent immunofluorescence in the brain. Skunks 23 and 24, killed on day 12 had immunofluorescent material in only a few acini in areas remote from the inoculation site. This is not consistent with direct spread among glandular cells and probably is due either to spread from the brain or via local neural networks within the gland. Previous studies indicate that spread of rabies virus to nonnervous tissues is unlikely to occur via blood or lymph (9). In a preliminary study, a skunk killed on day 15, had immunofluorescence in the gland restricted to the region of the inoculation site. Although the brain was positive by the fluorescent antibody test, extremely small amounts of immunofluorescent material were detected. Virus was not isolated from the brain of this skunk by intracerebral inoculation of weanling mice. Limited intrinsic cell-to-cell spread of virus may have occurred in this skunk. Slight immunofluorescence was detected in the brain so that a neural route cannot be ruled out. Even assuming direct infection from the inoculum, the time required for development of this regional infection suggests that spread among epithelial cells would not account for the widespread infection of glandular tissue seen in many naturally occurring cases of rabies. The only previous report of direct inoculation of rabies virus into the salivary gland was a study by Bertarelli (12) to determine infectivity of rabies virus given by this route. It contained no information on spread of virus in the gland.

The distribution of rabies antigen in skunk 30 suggests that, in this skunk, centripetal migration of virus occurred in sympathetic nerves, first from the right submandibular salivary gland to the right superior cervical ganglion and then to the thoracic spinal cord. Probably infection then spread to the left superior cervical ganglion and to the brain. The much more extensive neuronal involvement in the right ganglion than in all other sites examined suggests that this ganglion became infected early in the disease. Interneurons could facilitate transneuronal dendroaxonal transfer of virus (21) among principal neurons resulting in widespread neuronal infection in this ganglion.

Our previous studies indicate that transneuronal dendroaxonal transfer of virus is an important mechanism in spread of virus in the CNS. The findings suggested that virus was synthesized in the perikaryon or dendrites at the site

TABLE IV. Experiment 2. Serum Neutralization Titers of Skunks Exposed by Direct Inoculation of the Submandibular Salivary Glands

	Postinoculation sample							
	1		2		3		4	
Skunk No.	Time	Titer[a]	Time	Titer	Time	Titer	Time	Titer
29	Day 7	—	Day 12	—	ND[b]		Day 20	—
30	Day 7	—	Day 12	—	ND		Day 20	—
31	Day 7	—	Day 12	—	Day 19	—	Day 25	—
32	Day 7	—	Day 12	—	Day 19	—	Day 25	—
33	Day 7	—	Day 12	—	Day 19	—	Day 21	0.50
34	Day 7	—	Day 12	—	Day 19	—	Day 26	42.9
35	Day 7	—	Day 12	—	Day 19	—	Day 31	1.26
36	Day 7	—	Day 12	—	Day 19	—	Day 47	1.65
							Day 53[c]	147

[a]Titer in international units (See Table III)
[b]Not done
[c]Sample 5, skunk No. 36

of budding and transfer (21). This mechanism is suitable for retrograde transfer of virus, i.e. opposite to the polarity of the synapse. It is apparent that this mechanism would be effective in the CNS. However, in the peripheral nervous system neuronal processes that innervate receptors and effectors are axons. Since axons are devoid of ribosomes, it is unlikely that the same mechanism accounts for release of virus from terminal axons of peripheral nerves in the salivary glands or other tissues. No report of details of this process was found. We consider the following as possible mechanisms of release of virions from terminal axons: 1) wallerian degeneration with release of virions from fragmenting axons; 2) centrifugal movement of matrix, entrapped ribosomes and rough endoplasmic reticulum along axons, synthesis of viral components in axon terminals, and assembly on the axolemma; 3) synthesis of viral components in the perikaryon, followed by centrifugal migration and assembly at axon terminals; 4) reverse esotropy of virions previously synthesized in the perikaryon.

The usefulness of the routine diagnosis of rabies by the fluorescent antibody test is based on the assumption that an animal with brain negative by this test would not have had virus in saliva. Obviously this is valid in the majority of cases and our results generally support this assumption, even with direct inoculation of submandibular salivary gland.

Several reports indicate that nonfatal rabies infections, including recovery from clinical signs may occur in various species. Although there is much less evidence to support the excretion of virus in nonfatal infection (carrier state), this apparently rare phenomenon has been reported in dogs (22,23,24, 25,26) and in vampire bats (27,28,29). The reports of a carrier state in vampire bats have not been supported by recent research (30). We do not know of any well documented reports of the carrier state in naturally infected animals in North America. Studies of the duration of extraneural rabies infection in muscle (31) suggest that infection as detected by immunofluorescence usually is not long lasting and probably is eliminated by the immune response. Bell stated that most reports of the carrier state originated in Asia and Africa and may imply accommodation between host and virus in those regions (23). The present lack of evidence of this phenomenon in North America suggests that it does not merit changes in public health measures.

The occurrence of serum neutralizing antibodies by day 7 in some of the skunks in experiment 1 suggests that the immune response was activated by virus in the inoculum since immunofluorescence was not detected in any tissue at this time. The early occurrence of antibodies probably was due to the large amount of virus administered since it did not occur in skunks given more diluted (10^{-3}) infected salivary gland suspension.

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