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The long incubation period in rabies: delayed progression of infection in muscle at the site of exposure

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Abstract The striped skunk *(Mephitis mephitis)* is a host of rabies in large areas of Canada and the United States. In each of two experiments, equal numbers of skunks in two groups were inoculated intramuscularly with low doses of a field strain of rabies virus (street rabies virus). In each experiment, skunks in one group surviving to 2 months were killed at this time and selected tissues were used for examination by the polymerase chain reaction (PCR) method or by immunohistochemistry for rabies antigen. Results of detailed examinations using PCR technology (experiment 1) indicated that muscle at the inoculation site contained viral RNA at 2 months postinoculation, when other relevant tissues on the route of viral migration and early entrance into the central nervous system were negative. The cellular location of virus/antigen, as determined immunohistochemically in experiment 2, was striated muscle fibers and fibrocytes. Our results indicate a major role of muscle (tissue) infection at the inoculation site in the long incubation period of rabies in skunks. These and related findings will be useful in rabies control and, if applicable to other species, will be relevant in postexposure treatment.

Key words Rabies · Long incubation periods · Skunk · Polymerase chain reaction · Immunohistochemistry

Introduction

The incubation period in rabies ranges from about 2 weeks to several months, and rarely even to years [4, 8, 12, 14–16, 25, 26, 31]. The pathogenesis of rabies generally includes deposition of virus (in nature via bites of a rabid animal) into tissues (skin, muscle) of a susceptible animal, migration of virus up peripheral nerves to the central nervous system (CNS), spread throughout the CNS, and centrifugal spread via peripheral nerves to infect some nonnervous tissues [5, 6, 18, 33, 34]. The location of virus during the long incubation periods has not been determined [27], but is considered to be at one or more sites en route to or in the CNS. Suggested locations include: nonspecified tissue at the inoculation site [1], muscle at the inoculation site [7, 22], spinal ganglia (M. M. Kaplan, personal communication) and the CNS [21]. Part of the difficulty in studying this aspect of the pathogenesis is the high frequency of short incubation periods in most of the laboratory animal-fixed virus models used for studying the pathogenesis. In previous studies, we demonstrated that skunks given low doses of a field strain of rabies virus (street rabies virus) had a relatively high proportion of long incubation periods [8], determined the specific spinal cord segments and dorsal root ganglia infected first after inoculation of the distal pelvic limb [9], and developed a method for inoculation of a specific muscle in skunks [7].

In the present study, two experiments were used for detailed examinations by polymerase chain reaction (PCR) and immunohistochemistry to determine the location of virus/antigen at 2 months postinoculation during the incubation period. In each experiment, two groups of skunks were inoculated with low doses of street rabies virus. Skunks in one group surviving to 2 months were killed and relevant tissues on the route of viral migration and early entrance into the CNS were used for examination by a PCR or streptavidin-biotin complex (SBC) method; skunks in a second group were kept as positive controls.

Materials and methods

Experimental animals

Striped skunks *(Mephitis mephitis)* were obtained from a supplier (Ruby's Fur Farm, New Sharon, Iowa). they were kept in stainless steel cages and given food and water ad libitum. The experiments followed the guidelines of the Canadian council on Animal Care.

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Table 1 Experiment 1. Location of viral genomic RNA in tissues of skunks killed at 62–64 days postinoculation, determined by polymerase chain reaction. ($MICLD_{50}$ mouse intracerebral lethal $dose_{50}$, *ADQ* abductor digit quinti)

Skunk number	Dose ($MICLD_{50}$)	Right ADQ muscle	Other tissues[a]
1	26	+	–
2	26	+	–
3	26	–	–
4	26	–	–
5	26	+	–
6	26	–	–
7	260	–	–
8	260	–	–
9	260	+	–

[a] Other tissues included: left ADQ muscle, spinal cord segment at C8, and at T10 and all spinal cord segments caudal to L1, and right and left spinal ganglia at L3, L4, L5 and S1. All RNA preparations were shown to support RT-PCR by amplification of a 267-base portion of cytoplasmic-actin mRNA

Table 2 Experiment 2. Immunohistochemistry for rabies antigen in tissues of skunks killed at 62–64 days

Skunk number	Dose ($MICLD_{50}$)	Right ADQ muscle	Spinal cord (L2 and L3)	Right spinal ganglia (at L3, L4, L5, S1 and S2)
10	105	–	+	+
11	105	–	–	–
12	105	–	–	–
13	105	+ 5EF[a, b]	–	–
14	1050	+ 2EF[a]	–	–
15	1050	+ 6EF[a, b]	–	–

[a] Number of extrafusal fibers that contained antigen. Approximate extent of antigen-containing regions in individual fibers in each skunk: skunk 13 (200, 250, 250, 350 and 900 μm), skunk 14 (450 and 1300 μm), skunk 15 (50, 50, 50, 250 and 800 μm)
[b] A few small groups of antigen-containing fibrocytes were in the right abductor digiti quinti *(ADQs)* of skunks 13 and 15. The fibrocytes were adjacent to or near infected myocytes (*EF* extrafusal fibres)

Experimental procedure

In experiment 1, the abductor digiti quinti (ADQ) muscle of the right pelvic limb of each of 32 striped skunks (6 months old) was surgically exposed and inoculated with 0.1 ml of a suspension of salivary glands from naturally infected skunks by a previously described method [7]. Most of the inoculum was injected into the proximal half of the muscle. The titer of virus in undiluted (10%) suspension was $10^{6.7}$ $MICLD_{50}$/ml. The skunks were divided into two main groups, A and B, each group consisting of eight skunks given 26 $MICLD_{50}$ and eight given 260 $MICLD_{50}$ of virus. Sixteen group B skunks (positive controls) were kept until they developed rabies or for 18 months. All skunks that developed rabies were killed and necropsied.

Of 16 group A skunks, seven developed rabies at or before 64 days PI. The nine surviving skunks (group A) listed in Table 1 were killed at 62–64 days. Separate sets of sterile instruments were used for collection of the various tissues (inoculation site and other relevant tissues on the route of viral migration and entrance into the CNS) to be used for the PCR method. The tissues and sequence of collection were as follows: proximal left ADQ, left spinal ganglia at lumbar (L)3, L4, L5 and sacral (S)1, right spinal ganglia of same segments, spinal cord caudal to L1 and segments at cervical (C)8 and thoracic (T)10, and proximal right ADQ. These samples were kept at –70° C until tested. The distal half of the right and left ADQs, the brain and the remainder of the spinal cord were fixed in Zn formalin, and selected tissues were used for immunohistochemistry for rabies antigen.

In experiment 2, 40 skunks (20 group A and 20 group B) were used. The procedure of inoculation was the same as in experiment 1 with 10 of each group given 105 $MICLD_{50}$ and 10 given 1050 $MICLD_{50}$. The titer of undiluted (10%) suspension was $10^{6.2}$ $MICLD_{50}$/ml. Of the 20 group B skunks (positive controls), two died from causes unrelated to rabies. The remaining 18 were kept for 12 months or until rabies clinical signs began. Of 20 group A skunks, 14 developed rabies (before day 64). The six group A skunks listed in Table 2 were killed at 62–64 days. At necropsy, tissues were collected and fixed in Zn formalin, dehydrated and embedded in paraffin. The right ADQ muscle was divided into five segments for embedding. The entire muscle was serially sectioned (at 5 μm perpendicular to the axis of the muscle) and sections at 50-μm intervals were used for immunohistochemistry for rabies antigen. Similarly sections at 50-μm intervals of the entire spinal cord segments at L2 and L3 and entire right spinal ganglia at L3, L4, L5, S1 and S2 were stained and examined.

PCR analysis

Total RNA (2 μg), recovered from selected tissues using a modification of the acid phenol/guanidinium method as described by Kamolvarin et al. [19], was used to direct cDNA synthesis primed with a sequence-specific oligonucleotide. Reverse transcription was performed in a 20-μl volume containing 50 mM TRIS-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 50 pmol primer, dNTPs each at 1 mM and 200 units M-MLV reverse transcriptase (Life Technologies) at 37° C for 90 min. After heating (90° C, 5 min), the reaction received 10 μl of PCR buffer [0.1 M TRIS-HCl (pH 8.8), 0.5 M KCl, 15 mM $MgCl_2$, 1% Triton X-100] and 50 pmol of opposite sense primer, and was brought to a final volume of 100 μl. *Taq* DNA polymerase (2 units) was added by hot start, prior to thermocycling (94° C for 1 min, 55° C for 1 min, 72° C for 2 min + 5 s autoextension, for 30 cycles). The primer pairs used for rabies viral RNA detection, based on rabies virus sequences described previously [23, 24], were: 5′-GCACGGAAAGGAGATAAGATCACCC-3′ (+) and 5′-ATA(AG)GAGTGAGGAACAGCTGTCTC-3′ (–) for negative sense genomic RNA targeted to the N gene, 5′-GGTTAGTCGAGCAGTAGGTAGATG-3′ (–) and 5′-TGTCGGTTATGTCACCACTACGTT-3′ (+) for positive sense RNA of both mRNA and replicative intermediate (G gene locus), 5′-CTTGTATACCCAGTTCATGCCCT-3′ (–) and 5′-GCAGTTAAAAATGAACCTTGATG-3′ (+) which target a sequence across the P-M intergenic region and would thus specifically amplify the positive sense replicative intermediate. PCR products were electrophoresed through agarose and transferred to HybondN membrane. Specific products were visualized by autoradiography following hybridization of the Southern blot with oligonucleotide probes; these were generated from oligonucleotides corresponding to internal PCR product sequences by end-labelling using [^{32}-P]-ATP and T4 polynucleotide kinase.

RT-PCR of cytoplasmic-actin mRNA was performed using primers 5′-ATGGAGGGGCCGGACTCGTCATAC-3′ (–) and 5′-CTCCATCATGAAGTGTGACGTGGA-3′ (+) directed to different exons of the gene. Reactions were performed as for rabies RT-PCR, except that 25 pmol of each primer was used and the cycling profile, run for 25 cycles, was 95° C for 1 min, 60° C for 1 min, 72° C for 2 min + 5 s autoextension. DNA products, which were reverse transcriptase dependent, were visualised on agarose gels by ethidium bromide staining.

Immunohistochemistry

Five-micrometer sections at 50-μm intervals of the above tissues in experiment 2 and of the distal portion of the right ADQ of experiment 1 were stained by an SBC method [3].

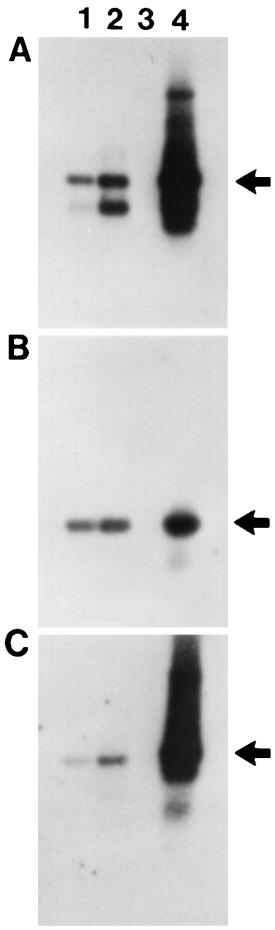

Fig. 1 Southern blot of products generated from animals 1 *(lane 1)* and 5 *(lane 2)* by reverse transcriptase polymerase chain reaction (RT-PCR) for negative sense genomic RNA at the N gene locus *(panel A)*, for positive sense RNA at the G gene locus *(panel B)* and for positive sense RNA spanning the P-M intergenic region *(panel C)*. Negative (no template) and positive (rabies-infected brain tissue) controls are shown in *lanes 3* and *4*, respectively. The sizes of all RT-PCR products were as predicted at 567 bp *(panel A)*, 296 bp *(panel B)* and 644 bp *(panel C)* (see *arrows*) as verified by a 100-bp DNA ladder marker (Life Technologies) run in parallel with all lanes. The greater signal strength for *lanes 1 and 2* in *panel B* indicates that positive sense sequence is present in these tissues predominantly as mRNA rather than as the replicative intermediate form detected exclusively in *panel C*

Results

In experiment 1, all selected tissues of the nine group A skunks (killed at 62–64 days PI) were tested by the PCR method for genomic RNA; those positive were also tested for messenger and replicative intermediate RNA. The right ADQs (proximal halves) of four of nine skunks were positive for genomic, messenger and replicative intermediate RNA (Fig. 1, Table 1). All other tissues (lumbar spinal ganglia, spinal cord caudal to the first lumbar segment, spinal cord segments C8 and T10 and left ADQ) in all nine skunks were negative. Although this demonstrates harborage of virus in muscle tissue at the inoculation site, the cells involved (myocytes) were suggested in only one skunk (skunk 7). (The distal half of the right ADQ was examined by the SBC method). This discrepancy may indicate the sparse amount of antigen present, but it could also be due to a disproportionate amount of virus/antigen in the proximal half of the muscle (since most of the inoculum was placed in this region). Eleven skunks in group B developed rabies, four before and seven after 64 days. Dose of virus and incubation periods were: 26 $MICLD_{50}$ (68, 139, 181 and 329 days) and 260 $MICLD_{50}$ (22, 22, 34, 62, 236, 269 and 272 days). Five skunks did not develop rabies within the 18-month observation period.

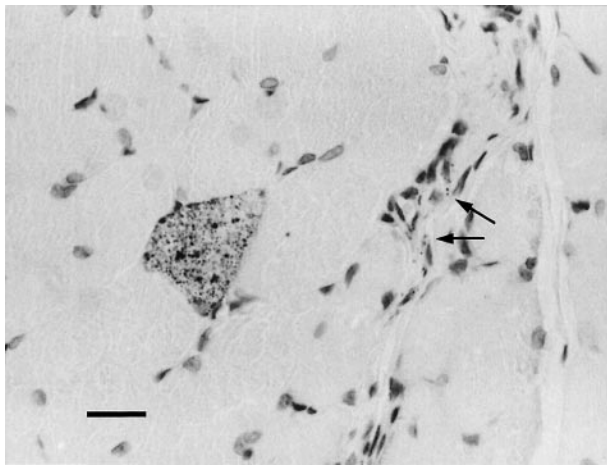

Fig. 2 Experiment 2. Streptavidin-biotin complex method on right abductor digiti quinti muscle (inoculation site). Stained rabies antigen in one extrafusal muscle fiber and in a few perimysial fibrocytes *(arrows). Scale bar* = 20 μm

In experiment 2, three of six group A skunks killed on days 62–64 had antigen in muscle fibers of the right ADQ. Two of the three also had antigen in a few fibrocytes (Table 2). Antigen occurred mainly as fine and course granules in the sarcoplasm of extrafusal fibers (Fig. 2); infected regions extended for 50–1300 μm (Table 2). Fibrocytes that contained antigen occurred in small groups that were adjacent to or near infected myocytes. Inflammatory cells were not detected near infected muscle fibers or fibrocytes. Rarely, a few small collections of inflammatory cells including small granulomas (associated with surgically displaced hair fragments and adnexa) were detected in the subcutis and adjacent epimysium of both positive and negative skunks. In the three positive skunks, antigen-containing muscle fibers were detected in only two of five blocks of muscle in two skunks and in only one of five in the third. This indicates that very thorough examination of the entire muscle is necessary (even when the precise inoculation site is known) to provide reasonable assurance of detecting a small number of positive fibers by immunohistochemistry. (The problems of similar detection in the naturally occurring disease would be virtually insurmountable.)

In skunk 10, the infection had progressed to the lumbar cord, right spinal lumbar ganglia and the brain. The number of antigen-containing neurons detected in right spinal ganglia at various levels of skunk 10 were: L3 (2), L4 (3), L5 (28), S1 (17) and S2 (1). The distribution of antigen-containing neurons was closely similar to that of skunks in a previous study [9], indicating that initial viral transit had occurred via nerves connecting the inoculation site and lumbar spinal cord. In experiment 2, 14 group B skunks developed rabies, nine before and five after 64 days. The dose of virus and incubation periods were: 105 $MICLD_{50}$ (22, 31, 31, 100, 114 and 119 days); 1050 $MICLD_{50}$ (22, 23, 28, 32, 39, 45, 72 and 112 days). Four survived the 12-month observation period. Rabies antigen

was not detected in the right ADQs of any of these four group B skunks killed at 12 months postinoculation.

Discussion

Our findings indicate that muscle at the inoculation site is a location of delay in progression of infection during the long incubation period. Virus and viral antigen are located in myocytes; infection of nearby fibrocytes is observed occasionally. Although primary infection of fibrocytes cannot be completely ruled out, our unpublished studies using high doses of inoculum indicate that early infection (within 1–2 weeks) of myocytes is much more common than infection of fibrocytes, suggesting that primary infection of fibrocytes is not an important element in long-term viral harborage. Also, each nondividing muscle cell could provide extensive cytoplasm to support continuous viral growth with less need for cell-to-cell transfer than would be likely to be the case with fibrocytes. The small number of muscle fibers affected (experiment 2) indicates very limited, if any, spread of infection among fibers. We did not find infected intrafusal fibers (muscle spindles) at this stage of infection. In previous studies of more advanced disease in skunks, intrafusal muscle fibers were commonly infected in muscle at and remote from the inoculation site, but only as a result of centrifugal viral transit in nerves from previously infected spinal cord or spinal ganglia [5].

The lack of detectable neutralizing antibodies associated with low doses of virus [8] and lack of inflammatory cells near antigen-containing muscle fibers suggests no, or minimal, induction of the immune response. These features would be consistent with long-term smoldering focal infection that is not eliminated by immune mechanisms. The final release of virions from infected fibers (with ensuing uptake by axon terminals) probably is due to budding on the sarcolemma or disintegration of individual muscle fibers subjected to long-term massive infection.

In rabies, the incubation period is highly variable in a wide range of host species [4, 8, 12, 36]. Except for dogs, most findings in host species rely heavily on experimental studies. The variable incubation period and especially the long incubation period are considered important in maintaining enzootic rabies [4, 36]. One study of the natural disease in dogs indicates that most incubation periods are less than 60 days with about 5% being over 60 days (60–150 days) [29]. Periods of several months have been recorded in the experimental disease [12]. Although very variable in humans, most cases have incubation periods between 1 and 2 months [17, 35]. About 15% are greater than 3 months [14] and periods up to several years have been documented [15, 16, 26, 31]. Cases with long incubation periods are more likely to lack a history of exposure making diagnosis more difficult [31]. The mechanisms of this delayed progression of the infection should be relevant in postexposure treatment – the only effective method of preventing disease after receiving an otherwise lethal dose of virus.

In models with relatively short incubation periods, infection of muscle fibers directly by virus in the inoculum was demonstrated in hamsters [22] and in skunks [7]. However, neither of these studies nor any other published study has provided convincing evidence that myocyte infection is an essential link in the pathogenesis. On the other hand, several studies have demonstrated direct uptake of virus by peripheral nerve and transit to the CNS without preliminary replication in non-nervous tissue [2, 10, 11, 20, 28].

We propose the following mechanisms to explain, at least partly, the wide variation in incubation periods in rabies. With high doses of virus, there is a high probability that there will be uptake of virions by both myocytes and axon terminals at the inoculation site. In this case, myocyte infection is predominantly incidental, the main course of infection proceeding from uptake of virions by axons and progression via retrograde axoplamsic flow directly to the CNS or to spinal ganglia (en route to the CNS). This scenario results in fairly short incubation periods generally within a narrow range. With increasingly lower doses of virus, there is a higher probability that virions will enter either axon terminals or muscle fibers (and, in some cases neither). This could then result in early transit to the CNS via axons of peripheral nerves or retention in muscle for varying periods of time before release and uptake by peripheral nerve axons, resulting in a wide range of incubation periods from fairly short to long, as noted in experiment 1 (22–329 days). This general relationship between dose and incubation period has been recorded in several other studies [8, 13, 30, 32].

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