Experimental Rabies in Skunks: Persistence of Virus in Denervated Muscle at the Inoculation Site

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ABSTRACT

Striped skunks (*Mephitis mephitis*) were inoculated into the denervated abductor digiti quinti muscle with street rabies virus. They were killed at various times after inoculation and several tissues were examined by immunofluorescence and light microscopy. Muscle at the inoculation site was examined electron microscopically. Rabies antigen was detected in muscle fibers first on day 7 and persisted until day 28. Light and electron microscopic lesions at the inoculation site included atrophic and degenerating muscle fibers and a few focal and regional endomysial accumulations of macrophages, lymphocytes and plasma cells. Scattered myocytes contained bodies of matrix, virions and anomalous tubular structures on electron microscopic examination. The results indicate that replication of rabies virus may occur in infected muscle fibers at the inoculation site until 28 days after exposure. This could contribute to variations in the incubation period for the first two to three months after exposure. However, the results do not support the contention that virus is contained in striated muscle cells throughout the long incubation periods.

RÉSUMÉ

Cette expérience consistait à inoculer le virus de la rage des rues dans le muscle dénervé *abductor digiti quinti* de mouffettes rayées (*Mephitis mephitis*). On sacrifia ensuite ces mouffettes à divers intervalles après leur inoculation et on examina plusieurs de leurs tissus par la technique d'immunofluorescence et au microscope photonique. On examina aussi le tissu musculaire du site d'inoculation, au microscope électronique. On commença à détecter de l'antigène rabique dans les fibres musculaires, le septième jour après l'inoculation, et il y persista jusqu'au 28^{e} jour. Les lésions décelées au site d'inoculation, par la microscopie photonique et électronique, incluaient de l'atrophie et de la dégénérescence des fibres musculaires; l'endomysium présentait par ailleurs quelques foyers d'accumulation de macrophages, de lymphocytes et de plasmocytes. La microscopie électronique démontra que quelques myocytes contenaient de la matrice virale, des virions et des structures tubulaires anormales. Les résultats de cette expérience révèlent que la réplication du virus rabique peut se faire dans les fibres musculaires infectées, au site d'inoculation, jusqu'au 28^{e} jour après l'injection. Ce phénomène pourrait contribuer aux variations de la période d'incubation, pour les deux ou trois premiers mois ultérieurs à la contamination. Ces résultats ne supportent toutefois pas l'hypothèse selon laquelle le virus rabique se retrouverait dans les cellules des muscles striés, d'un bout à l'autre des longues périodes d'incubation.

INTRODUCTION

The incubation period in naturally occurring rabies may vary widely (from approximately two weeks to more than one year); however, it is usually in the range of two weeks to three months. In cases with short incubation periods, virus at the inoculation site probably enters peripheral nerves soon after exposure and migrates directly to the central nervous system (CNS) without preliminary proliferation in nonnervous tissue. Presumably during intermediate or long incubation periods viable virus is retained in one or more sites *en route* to or within the CNS. However, the precise location of viral harborage during these long incubation periods has not been reported. Recently we reported infection of myocytes at the inoculation site of skunks given street rabies virus and suggested that virus may be retained in infected myocytes during intermediate or long incubation periods (8, 9).

To study the persistence of myocyte infection, the abductor digiti quinti muscle was denervated immediately before inoculation with street virus. At various times after inoculation this muscle was examined by immunofluorescence, light, and electron microscopy.

MATERIALS AND METHODS

VIRUS

A 10% suspension of salivary glands from naturally infected

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skunks was prepared by homogenization with diluent (0.01 M phosphate buffered saline, pH 7.4, containing 20% inactivated horse serum, 1000 IU penicillin and 2 mg streptomycin/mL) and centrifugation at 600 g for 15 minutes. The dilution containing one mouse intracerebral lethal dose 50 ($MICLD_{50}$)/0.03 mL was $10^{-6.5}$.

EXPERIMENTAL ANIMALS

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

EXPERIMENTAL PROCEDURE

In preliminary studies, denervation of the inoculation site by section of the sciatic nerve in the distal third of the femur resulted in severe self-mutilation of the foot. Subsequently we sectioned the lateral plantar nerve about 2 cm distal to the hock joint. This interrupted the nerve supply to the abductor digiti quinti muscle, but retained innervation of skin and subcutaneous tissue, thereby preventing postoperative self-mutilation.

Each of 28 skunks was anesthetized with Ketaset[2] (10 mg/lb) and Atravet[3] (0.25 mg/lb). Approximately 0.5 cm of the right lateral plantar nerve was resected through an incision approximately 2 cm distal to the hock joint. The right abductor digiti quinti muscle of 26 principals was inoculated with 0.3 mL of a 10% suspension of salivary glands from naturally infected skunks (9). The inoculum contained approximately $10^{6.5}$ $MICLD_{50}$'s. Two controls were inoculated with the same amount of suspension of salivary glands from noninfected skunks. The controls were killed on day 21. The principals were killed at various times after inoculation (Table I), and the following tissues were frozen and stored in liquid nitrogen for later immunofluorescence studies using a hamster conjugate (9): proximal and distal portions of the abductor digiti quinti muscles of the right and left pelvic limbs, the long digital extensor of the right pelvic limb, spinal cord at L-5, and medulla oblongata. A 1 mm thick equatorial section of the right abductor digiti quinti muscle was fixed in a solution of 1% paraformaldehyde and 1.5% glutaraldehyde in 0.2 M phosphate buffer, pH 7.4. This tissue was postfixed in 1% osmium tetroxide, dehydrated in alcohols and embedded in Epon. Thick cross sections of the entire muscle were cut, on a sliding microtome,[4] and stained with toluidine blue. Selected areas of blocks and/or large sections were processed for electron microscopic examination.

Other tissues including brain, spinal cord, various cerebrospinal ganglia and visceral organs were fixed in 10% neutral buffered formalin, and embedded in paraffin. Sections were cut at 6 μm and stained with hematoxylin and eosin.

RESULTS

Eight skunks developed clinical rabies, and thus were not used for studies of duration of myocyte infection. They are described at the end of the results section.

The skunks used to determine the duration of myocyte infection (Table I) did not develop clinical rabies, and at the time of euthanasia had no immunofluorescence for rabies antigen in the brain or lumbar spinal cord. Infection of intrafusal and extrafusal fibers may occur as a result of centrifugal migration of virus following CNS infection (9). In the skunks listed in Table I, this mechanism has been ruled out by negative immunofluorescence findings in the CNS and in muscles remote from the inoculation site.

The skunks killed on day 7 and later had mouse serum neutraliza-

TABLE I. Rabies Virus Infection of Denervated Muscle (right pelvic abductor digiti quinti) in Skunks

Skunk No.	Time of Euthanasia	Mouse Serum Neutralization Titer	Immunofluorescence
1	4 hr	—	+[a]
2	4 hr	—	+[a]
3	4 da	—	—
4	4 da	—	—
5	7 da	1:10	+
6	7 da	1:20	+
7	14 da	1:40	++
8	14 da	1:40	++
9	21 da	1:16	++
10	21 da	1:16	++
11	28 da	1:21	+
12	28 da	1:49	—
13	42 da	1:11	—
14	42 da	1:40	—
15	70 da	1:15	—
16	70 da	1:11	—
17	98 da	1:40	—
18	98 da	1:20	—

[a]Immunofluorescence in the endomysium only

[1]Ruby's Fur Farm, New Sharon, Iowa.
[2]Ketamine hydrochloride, Rogar/STB, London, Ontario.
[3]Acepromazine maleate, Ayerst Laboratories, Montreal, Quebec.
[4]R. Jung AG, Heidelberg, Germany.

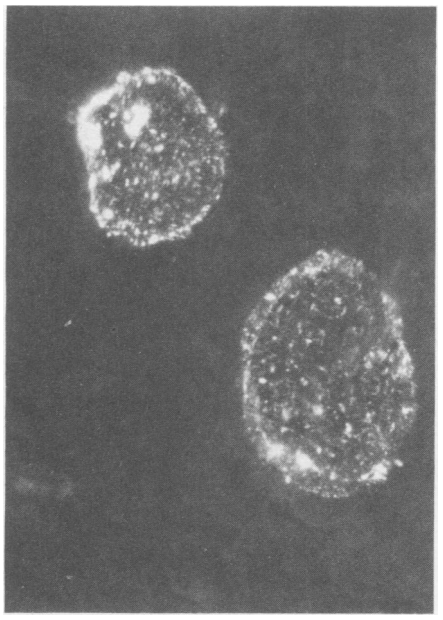

Fig. 1. Right abductor digiti quinti muscle. Immunofluorescence in two myocytes. Twenty-one days postinoculation. X225.

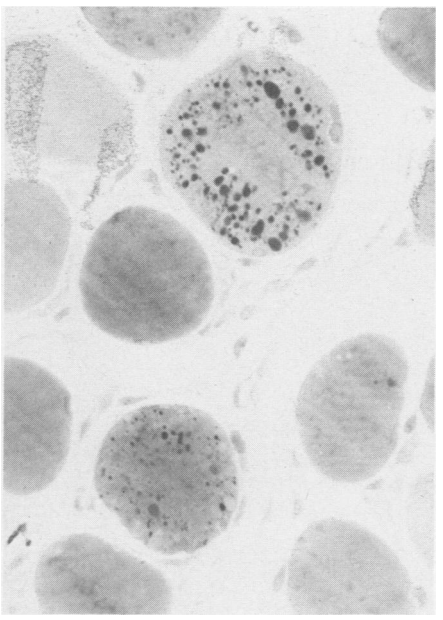

Fig. 2. Skunk 9, 21 days postinoculation. Dark blue bodies in sarcoplasm of muscle fibers of right abductor digiti quinti muscle. Toluidine blue. X403.

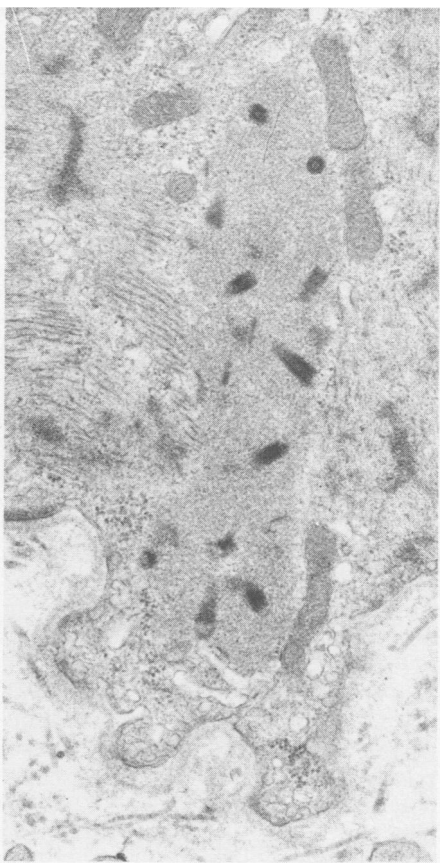

Fig. 3. Skunk 8, 14 days postinoculation. Body of matrix and virions in muscle fiber of right abductor digiti quinti muscle. Uranyl acetate and lead citrate. X20,395.

tion titers $\geqslant$1:10 (Table I). Fine granules of immunofluorescent material were in the endomysium of the right abductor digiti quinti muscle (inoculation site) at four hours (Table I). Extrafusal muscle fibers contained immunofluorescent material on days 7, 14, 21 and 28 (Table I, Fig. 1). Usually, affected fibers occurred singly or in groups of two to four; more fibers contained antigen on days 14 and 21 than on days 7 and 28. On day 28, fluorescence was detected in only four fibers of one of the two skunks. There were fine granules usually throughout the entire cross section of the fiber; in longitudinal or oblique sections the granules were aligned with the myofilaments.

Focal and regional endomysial accumulations of inflammatory cells occurred in the abductor digiti quinti muscle of animals killed at four hours and on all the days of euthanasia up to and including day 70. Initially there were neutrophils and a few macrophages; later (days 7-70), most of the inflammatory cells were macrophages, lymphocytes and plasma cells.

There were a few scattered atrophic fibers beginning on day 14; they were most numerous on day 70 and later. Degenerating fibers (pale and/or fragmented) were infrequent but a few were seen on day 4 and thereafter. In three skunks, there were one or more degenerating fibers containing round or oval homogeneous dark blue bodies (Fig. 2).

Fig. 4. Skunk 9, 21 days postinoculation. Granular bodies of various sizes in sarcoplasm between myofibrils. Uranyl acetate and lead citrate. X20,775.

Electron microscopically, extrafusal fibers of the right abductor digiti quinti muscle contained viral matrix, anomalous tubular structures and a few virions on day 14 (Fig. 3). These structures were morphologically similar to those observed in intact skunks (9). On days 14 and 21, many fibers contained finely granular and filamentous bodies (Fig. 4). They were generally similar to viral matrix seen previously (9), but varied in density of staining (Fig. 5); some were partially or completely surrounded by a membrane.

A few skunks (numbers 8, 9, 11 and 15) killed on days 14, 21, 28 and 70, had several fibers containing sarcoplasmic crystalloid structures (Fig. 6). The crystalloids consisted of intersecting rows of round profiles (27/nm in diameter) joined by fine filaments (Fig. 7). The rows intersected at 60°. The average center to center distance of these round profiles in each of the three rows in one structure (Fig. 7) were 64.6 nm, 63.1 nm and 60.0 nm. Most of the crystalloids had the same general structure in all sections and were similar to those previously seen (9). In the present study, they occurred free in the cytoplasm and were frequently adjacent to vesicular or dense bodies, or mitochondria. We have detected these structures in denervated muscle in control skunks but not in control intact skunks.

Degenerating fibers, seen at various times after inoculation, were characterized by vacuolation of sarcoplasm, fragmentation and clumping of myofilaments, swelling and vacuolation of mitochondria and accumulations of dense bodies. Structures similar to the homogeneous dense blue bodies seen by light microscopy (Fig. 2) consisted of irregular layers of membrane and homogeneous dense material.

Approximately 30% of the skunks inoculated into the denervated abductor digiti quinti muscle developed rabies. The average incubation period was 22 days, compared to 17 in intact skunks inoculated with the same dose of virus (unpublished studies). Immu-

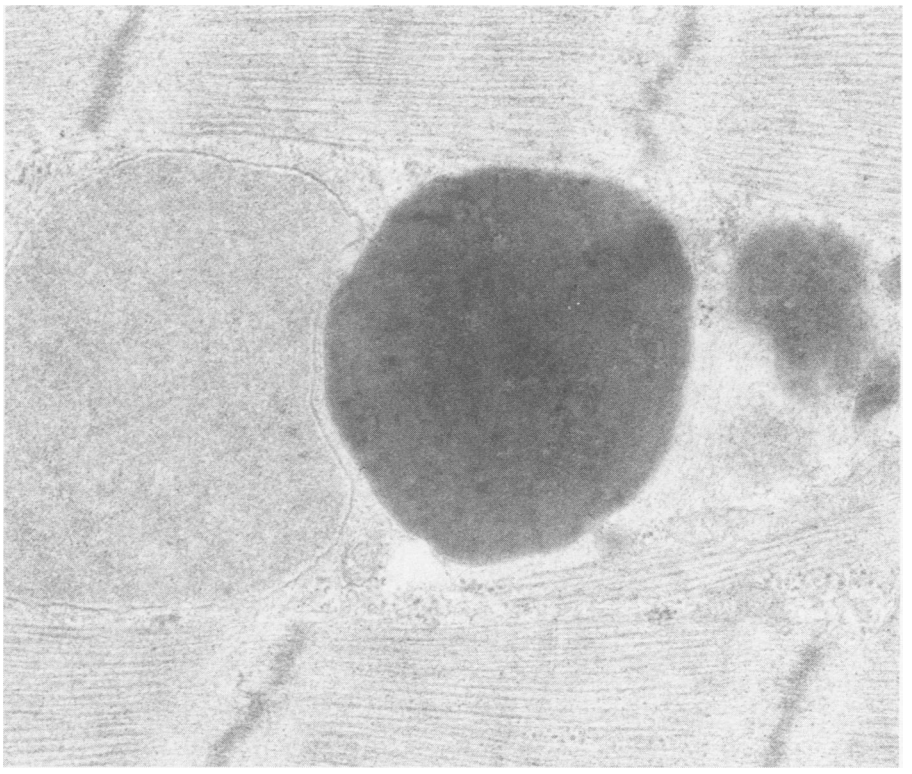

Fig. 5. Skunk 9, 21 days postinoculation. Granular body surrounded by a membrane and adjacent to a dense body. Uranyl acetate and lead citrate. X31,165.

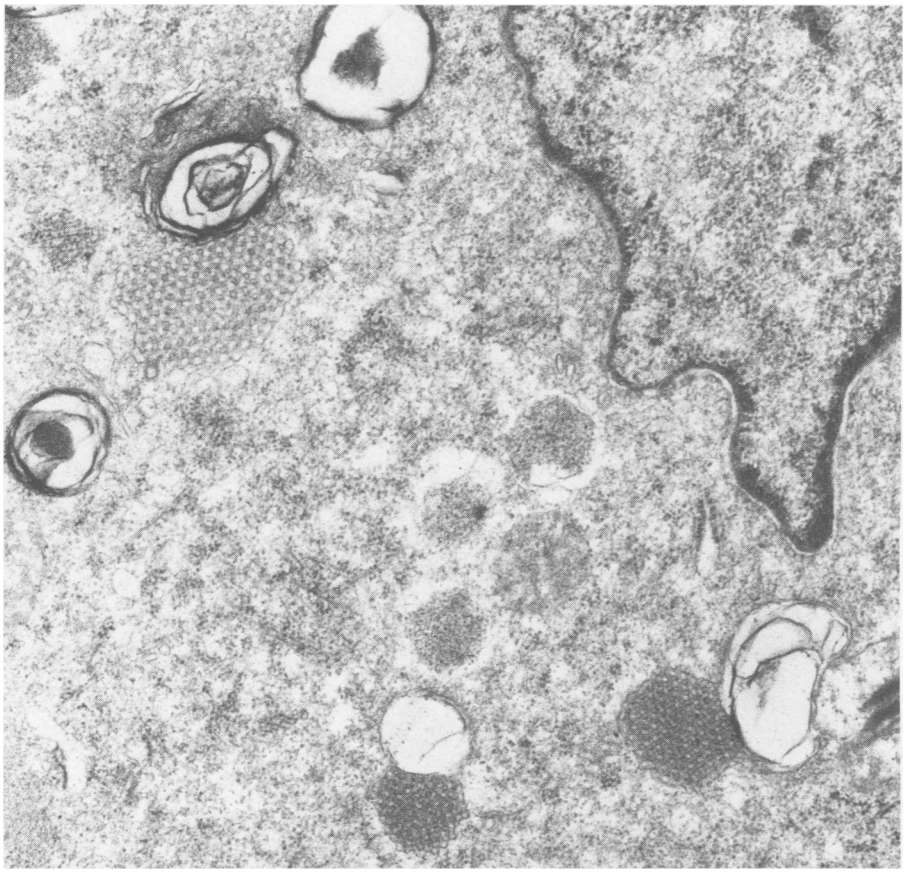

Fig. 6. Skunk 15, 70 days postinoculation. Several crystalloid structures in sarcoplasm adjacent to nucleus of a myocyte. Uranyl acetate and lead citrate. X20,775.

nofluorescence was detected in the spinal cord and brain stem in all of these skunks, and some had antigen in the right and/or left abductor digiti quinti muscle. Light microscopic lesions in the CNS were similar to those previously reported (9).

DISCUSSION

The development of rabies in an animal exposed by peripheral inoculation is generally considered to involve centripetal migration of virus in peripheral nerves to the CNS, spread through the CNS, and centrifugal migration of virus along peripheral nerves to infect some nonnervous tissues. Delay in the progression of infection at one or more sites *en route* to or within the CNS could result in delay of the onset of clinical signs. These sites include nonnervous tissue at the inoculation site, peripheral nerves, cerebrospinal and autonomic ganglia, and the CNS. Recent studies indicate that myocytes at the inoculation site of skunks (9) and hamsters (21) may be infected by virus in the inoculum, and in mice virus may be retained at the inoculation site at least for 18 days postinoculation (3). These findings suggested that retention of virus in myocytes may be a mechanism in variations in the length of the incubation period (9).

Our findings indicate that extrafusal muscle fibers at the inoculation site can contain antigen from 7 to 28 days postinoculation. The electron microscopic findings are compatible with rabies virus replication and indicate that these muscle fibers could be a source of infection for the CNS. The short duration (28 days) of myocyte infection, as detected in this study, probably was not due to denervation. The number of fibers affected, the pattern of immunofluorescence and electron microscopic characteristics did not differ from myocyte infection in intact skunks (9).

Although myocyte infection could contribute to variations in the length of the incubation period during the first two to three months after inoculation, the results do not support the contention that harborage of virus in myocytes accounts for the long incubation periods. We do not know the reasons for termination of myocyte infection, as detected by immunofluorescence and electron microscopy, on day 28. Probably the immune response was a factor. Further studies of the duration of myocyte infection with experiments to suppress or avoid the immune response are warranted.

The crystalloid structures in muscle of four skunks were identical to the structures previously reported in infected muscle of intact infected skunks (9). Although they may be adjacent to viral matrix, most are adjacent to degenerating organelles or occasionally are in areas of morphologically normal sarcoplasm. Structures described as crystals, crystalline arrays, or cytoplasmic inclusions have been described in otherwise normal human skeletal muscle (10, 14), in polymyositis in man (1, 11, 12, 16), in a case of postinfectious polyradiculoneuropathy in man (19), in experimental Coxsacki A virus myositis in mice (23), in diabetic amyotrophy and neuropathy (24) and in subacute myelo-optic neuropathy (22). Crystalline formations have also been seen in tissue culture cells infected with Coxsackie virus (17). The crystalloids in our skunks did not resemble any of the above structures but were similar to crystalloids reported in human atrophic muscle fibres (25). The occurrence of these structures in denervated muscle of noninfected control skunks and their frequent proximity to degenerating cellular organelles in infected skunks suggests that they are nonspecific lesions associated with atrophy and/or injury to muscle cells. As demonstrated in electron microscopic studies of multiple sclerosis brain, there is need for caution in interpretation of particles in lesions as viruses (18).

The loosely granular bodies depicted in Figure 4 were interpreted as bodies of matrix. Although the granular material was not as compact as that in most rabies inclusions and no virions were associated with these structures, the distribution (aligned with myofibrils) was similar to the

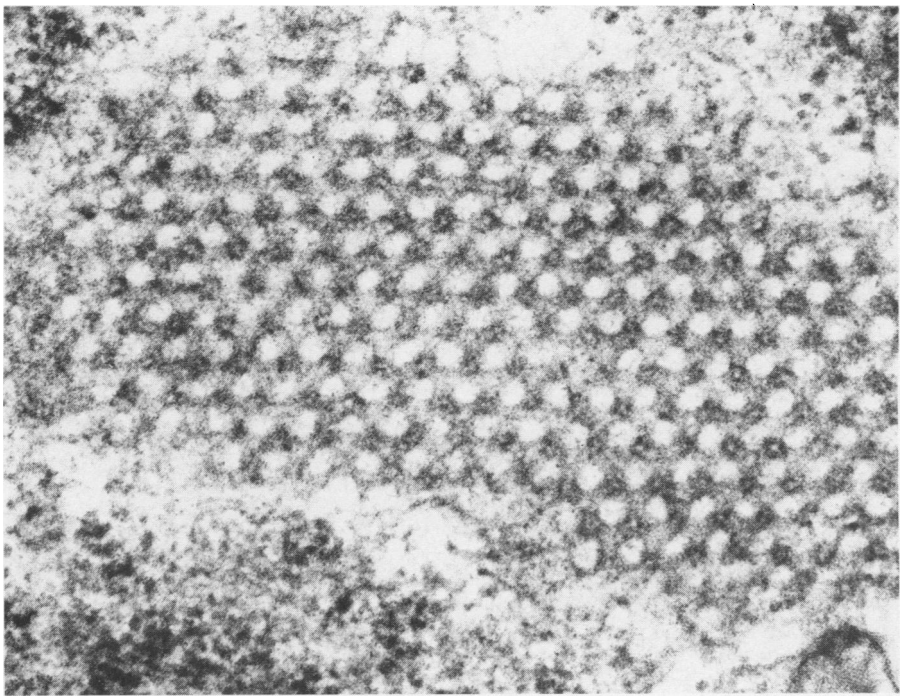

Fig. 7. Skunk 15, 70 days postinoculation. Crystalloid. Intersecting rows of round profiles joined by fine filaments. Uranyl acetate and lead citrate. X83,115.

distribution of particles of immunofluorescent material. Some granular bodies were enclosed by membranes and were adjacent to dense bodies (Fig. 5). This lesion may represent intracellular degeneration of viral nucleocapsid in morphologically intact fibers.

In regions with enzootic fox, skunk, raccoon or bat rabies, clinically normal animals may have serum neutralization titers against rabies (2, 6, 15, 20, 26). Gough and Niemeyer (15) stated that antibodies against rabies virus could be detected by several serological procedures in the sera from many skunks in a study in Iowa. The prevalence of SN titers in raccoons may vary widely, depending on whether samples are collected during an enzootic or epizootic (20). The foxes studied were not held to determine whether or not these serologically positive animals would develop rabies (26). Our studies in skunks indicate that virus sequestered at the inoculation site can induce SN titers without disease. This "abortion" of infection at the inoculation site probably contributes significantly to the SN titers in normal appearing animals in enzootic or epizootic areas.

Approximately 30% of the skunks inoculated into the denervated abductor digiti quinti muscle developed rabies. This may have been due to migration of virus along intact nerves in the skin and subcutis. In four studies of the transit of rabies virus from the inoculation site to the CNS, neurectomy or amputation performed at approximately the same time or several days before the inoculation of virus did not prevent rabies in all the experimental animals (4, 5, 7, 13). Dean and coworkers demonstrated that the sparing effect of neurectomy was less with hamsters than with mice or rats, was less with young than mature mice and rats and varied inversely with dose of virus (13). They attributed mortality in neurectomized animals to blood-borne infection from the site of inoculation, and suggested that blood-borne infection in nature is probably the exception rather than the rule. Bijlenga and Heaney (7) found that a few mice inoculated with street virus and simultaneously neurectomized or treated with vinblastine proximal to the inoculation site died. They suggested that virus is these cases was transported to the CNS by a lympho-haematogenous route activated by the surgical procedure (7). Although in most reports the frequency of rabies deaths in neurectomized animals is low, the consistency of occurrence in several studies indicates that the phenomenon merits further study, especially in very young animals.

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