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Immunofluorescent Examination of the Skin of Rabies-Infected Animals as a Means of Early Detection of Rabies Virus Antigen

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Correlations were made on immunofluorescence positivity to antirabies conjugate between cranium-derived nerve fibers in skin and traditional samplings of brain tissue from several species and illness categories of animals with naturally acquired rabies. The overall correlation of results from all categories was about 98% (*n*, 104) for those that were brain positive and 100% (*n*, 99) for those that were brain negative. Some animals that ultimately developed rabies were found to have immunofluorescence-positive results 2 or more days before the onset of clinical signs in both natural and experimental infections. The percentage of those with positive skin immunofluorescence results increased as the onset of symptoms approached. From the midcourse period of illness to death, the correlation between skin and brain approached 100%. Different vaccines, commonly given to prevent rabies and other diseases of dogs and cats, were administered to groups of mice and were found to not produce false-positive results when their skin was examined by immunofluorescence for rabies virus antigen. These data suggest that examination of surgical biopsy specimens by immunofluorescence for rabies virus antigen is a useful and reliable diagnostic tool to evaluate the rabies status of biting dogs or cats, or to confirm a clinical diagnosis of rabies in the species tested. The biopsy evaluation of any other species as a means of assessing bite risk is not suggested by these data.

Clinical rabies has historically been regarded as being uniformly fatal, in part because a laboratory-confirmed diagnosis of rabies was made only when brain tissue was available. However, Pasteur, as well as investigators both before and after his time, mentioned recoveries from rabies (1); abortive and recovered rabies is now a well-documented phenomenon. Bell (1) listed criteria for proving that an apparently healthy animal or person had had an infection with rabies virus. These criteria are (i) a high titer of rabies neutralizing antibody in the cerebrospinal fluid (greater than the normal 1:100 physiological ratio with the blood serum), (ii) a positive immunofluorescence (IF) of smears of corneal epithelium, and (iii) isolation of rabies virus from the saliva of an animal that does not succumb. Any one of these criteria is sufficient to justify a diagnosis of rabies.

IF examination has been used to detect rabies virus antigen in the muzzle skin of mice well before the onset of disease after they had been inoculated with street virus (11) and in the muzzle skin of skunks incubating naturally acquired rabies (3). Positive IF examinations have confirmed the presence of rabies virus antigen in the face skin of street virus-inoculated dogs on their first day of clinical illness (Blenden, unpublished data, 1983). Examination of IF-stained skin sections has been used for identifying the presence of rabies virus antigen before and at the time of death in the following species: dogs, cats, cattle, foxes, skunks, raccoons, rhesus monkeys, mongooses, horses, several species of bats, goats, pigs, and humans (2–6, 14; D. C. Blenden, J. Am. Vet. Med. Assoc. **165**:735, 1974; D. C. Blenden, CDC Symposium on Advances in Rabies Research, September 1976; D. C. Blenden, J. Am. Vol. Med. Assoc. **179**:265, 1981). A total of 234 skin specimens from six species were reexamined by IF after having been stored at −20°C for several years and gave 93.4% correlation with previously obtained results (12).

The use of IF examination of skin specimens obtained by surgical biopsy or at necropsy

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seems to have great potential for (i) laboratory confirmation of an early diagnosis during the clinical phase of rabies, (ii) laboratory confirmation of those infected animals not destined to die and which would otherwise escape detection, (iii) evaluation of a biting animal for the benefit of the person bitten while preserving the life of the animal, and (iv) acquisition of specimens from the field in species where the shipment of an entire head or brain is difficult or craniotomy is difficult (e.g., cows).

This report conveys results of three separate experiments to enhance the confidence placed in the use of skin for diagnosis and prediction of onset of rabies, and to test the effect of several clinically relevant variables upon results of the test. The objectives of these experiments were (i) to determine the degree of correlation of results obtained by IF examination of nerve fibers of skin with traditional examination of brain tissue from individuals naturally infected with rabies both at necropsy and biopsy stages, (ii) to determine the temporal relationship between skin IF positivity and onset of disease, and (iii) to determine the influence of various commonly used virus vaccine antigens on skin IF positivity.

MATERIALS AND METHODS

Matched skin and brain specimens were obtained with the extensive cooperation of many health departments and agencies, both inside and outside the United States. Many specimens were obtained within the state of Missouri. The species represented in this study were dogs, cats, cattle, swine, horses, foxes (grey and red), striped skunks, raccoons, mongooses, and bats (several species). A total of 232 separate animals, 116 having rabies IF-positive brain and 116 having rabies IF-negative brain, were examined.

The second and third experiments were done on animals exposed to rabies in the Montana laboratory of J.F.B. and sent to Missouri for examination. Mice were prepared for the second experiment by using several different original or first-mouse-passage street virus isolates inoculated via intracranial, intramuscular, and intraperitoneal routes. Randomly selected animals from each group were killed and quick-frozen at various predetermined times in relation to the expected onset of illness, based on previous determinations of the incubation period (1, 3, 5, 7, 10, and 15 days post-inoculation). The inoculated mice were coded together with uninoculated control mice and shipped frozen. Upon receipt by the University of Missouri laboratory, 3 to 4-mm pieces of muzzle skin were removed, processed, and examined as discussed below. The results were calculated with the number of skin-positive mice as the numerator and the number examined as the denominator for each time datum. The percentage of skin-positive mice for each day before, during, and after the onset of rabies, as well as for those mice surviving acute infection, was recorded on a histogram (see Fig. 2). A total of 337 test and control mice were studied, entailing the examination of 1,685 slides and 3,370 sections.

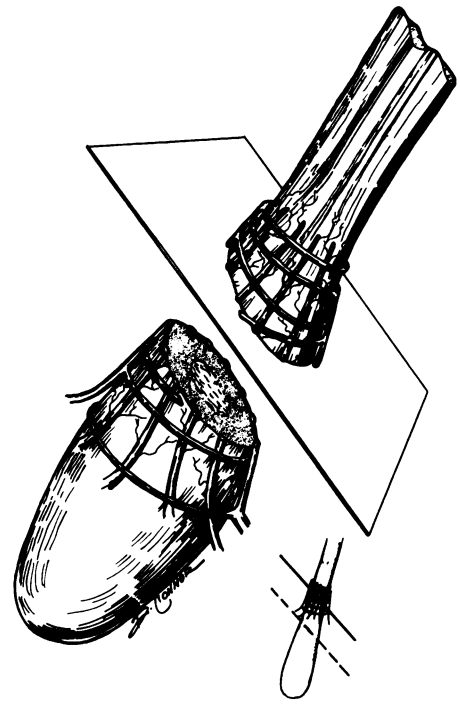

FIG. 1. Diagramatic oblique section of hair follicle showing area with the highest concentration of parafollicular nerve fibers (insert shows how easily this can be missed).

The third experiment was designed to test whether various virus vaccine antigens used commonly in dogs or cats might produce false-positive results. Groups of mice were given various vaccines, and random mice were selected, killed, and fast-frozen at the time of and up to 15 days after vaccine administration. Rabies-infected mice and those injected with distilled water only were included as controls. The mice were labeled by code and shipped frozen in dry ice from Montana to the University of Missouri. Interpretations were again carried out in the blind. A total of 245 mice were studied, with 1,225 slides and 2,450 sections. Of the total, 210 of 245 were given one of eight different vaccine antigens, and the remaining 35 of 245 were either uninoculated or rabies-inoculated controls. Both living attenuated and killed virus vaccines were used (see Table 4).

The skin specimens were trimmed as needed, and hair was snipped of with fine scissors. The skin specimens were mounted with the dermis upward on brass cryostat buttons with a commercial mounting medium (O.C.T. compound, Tissue Tek II; LabTek Products, Naperville, Ill.). The buttons were then placed into the cryostat chamber (I.E.C., CTF Microtome-Cryostat; International Equipment Co., Needham Heights, Mass.) and allowed ample time to solidify at −20°C. Oblique sections 10 μm thick were cut with the sharpest blade possible. At least two sections, consecutive when possible, were placed on precleaned glass slides. The level from which sections were cut is important and is graphically illustrated in Fig. 1. Five

TABLE 1. Summary of results of immunofluorescence examination of skin and brain of naturally infected animals (excluding bats) for rabies virus antigen

Brain result (*n*)	No. positive/total examined (% positive)	
	Antemortem[a]	Postmortem
IF positive (104)	68/69 (98.6)	34/35 (97.1)
IF negative (99)	0/76 (0)	0/23 (0)

[a] In most cases, antemortem specimens were secured at or beyond the midpoint of the course of illness.

slides were prepared from each tissue block, each slide with at least two sections. After fixing in acetone at −20°C for 20 min, the slides were washed in buffered water (pH 7.6) for 1 min to fix the tissue and remove residual mounting medium. The slides were then air dried, and each tissue section was circled with a wax pen. Commercial fluorescein isothiocyanate-labeled anti-rabies conjugate (BBL Microbiology Systems, Cockeysville, Md.) was used at a 1:150 dilution in a standard direct staining procedure (7) with Evans blue as a counterstain.

The microscopic examination using UV epi-illumination (Leitz Ortholux II with HBO 100-W mercury lamp, epi-illumination, and KG-1 heat absorption, 2xKP490 (KP500) excitation, and K45 suppression filters) was initially done under low-power magnification to locate large and tactile hair follicles and then changed to 970X magnification with nonfluorescing immersion oil directly on the tissue sections. Examination was concentrated on the periphery of the germinal portion of the hair follicles, where the greatest concentration of nerve follicles is known to be located (11; Fig. 1). The presence of distinct rounded dots of apple green fluorescence in the nerve plexus surrounding the follicle (nerve fibers are less common in other areas of the tissue) indicated a positive reaction; the absence of such fluorescence in the inhibition and negative control sections indicated specificity.

RESULTS

Of those animals that yielded positive results upon examination of brain tissue, 98% (102 of 104) also had a positive result on examination of the skin, and 2% (2 of 104) had negative skin results (Table 1). Of those animals giving a negative result upon examination of the brain, 100% (99 of 99) also had negative skin results. A total of 29 bats of mixed species were studied,

TABLE 2. IF examination of brain compared with ante- and postmortem acquired skin of naturally infected animals for rabies virus antigen

Brain IF (*n*) and species	No./total tested			
	Antemortem		Postmortem	
	Skin positive	Skin negative	Skin positive	Skin negative
Positive (116)				
Dogs	13/14	1[a]/14	2/3	1[b]/3
Cats	7/7	0/7	2/2	0/2
Cows	1/1	0/1	4/4	0/4
Horses	1/1	0/1	1/1	0/1
Pigs	0/0	0/0	1/1	0/1
Skunks	24/24	0/24	9/9	0/9
Foxes[c]	22/22	0/22	8/8	0/8
Racoons	0/0	0/0	4/4	0/4
Bats[c]	0/0	0/0	7/12	5/12
Mongooses	0/0	0/0	3/3	0/3
Negative (116)				
Dogs	0/33	33/33	0/4	4/4
Cats	0/7	7/7	0/1	1/1
Cows	0/7	7/7	0/1	1/1
Horses	0/11	11/11	0/2	2/2
Pigs	0/0	0/0	0/2	2/2
Skunks	0/4	4/4	0/1	1/1
Foxes	0/14	14/14	0/3	3/3
Racoons	0/0	0/0	0/9	9/9
Bats	0/0	0/0	1/17	16/17
Mongooses	0/0	0/0	0/0	0/0

[a] This specimen was submitted by a laboratory out of the country; brain and skin were separate. A labeling error could not be ruled out.

[b] This specimen, submitted by the Wisconsin State Laboratory of Hygiene, was from a dog exhibiting a typical clinical syndrome of rabies and had limited fluorescing antigen even in the brain.

[c] Both gray and red foxes were represented. Bats included mixed species, including 3 big brown bats, 3 little brown bats, 1 silver-haired bat, and 22 unidentified.

TABLE 3. IF examination for rabies virus antigen of antemortem acquired skin of animals with undiagnosed illness and comparison with ultimate brain examination

Brain result	n	Skin result	
		No. positive/ total (%)	No. negative/ total (%)
IF positive	70	68/70 (97.1)	2/70 (2.9)
IF negative	63	0/63 (0)	63/63 (100)

but were excluded from the summary data in Table 1. Because there is widespread discrepancy between the results of brain and skin from bats, inclusion would produce unnecessary bias. Table 2 gives a detailed view of individual species tested, including bats, and the numbers examined, both positive and negative. Bats excluded, the correlation of the results obtained by examining skin and brain was very high.

Since many of the skin specimens were secured from animals having illness, requiring differential diagnosis from rabies, the skin biopsy IF examination was used as a means of antemortem detection (Table 3). A total of 133 sick animals of several species was so examined. Of those that ultimately proved to have a rabies-positive brain and a confirmed diagnosis of rabies, 97.1% (68 of 70) were earlier found to have positive skin results. Of those that were ultimately proven to be nonrabid, 100% (63 of 63) were found to have negative skin results.

In the second experiment, there were no mouse skin positive results 5 and 6 days before onset, but on prodomal day 4, 6 of 29 mice (21%) were positive. Reactors increased more or less linearly until the first day of symptoms of rabies when 55 of 55 (100%) were skin positive (Fig. 2). During their illness, 100% of the mice were consistently positive until all but 40 of those which had developed clinical rabies had died. Of the 40 (40 of 237 sick or 17%) that survived, 11 of 19 (58%) had positive skin between 6 and 10 days after onset. On days 11 and 12 after onset, there was a total of 21 mice which had sickened and recovered; all of them had negative skin results. Of a total of 19 mice that were inoculated, but did not develop symptoms, 3 had positive brain results; 2 of these also had positive skin results. There were 39 control mice injected with water; all had totally negative skin.

TABLE 4. Effect of prior administration to mice of rabies and other virus vaccines on skin positivity by IF with antirabies conjugate

Vaccine	No. positive/ no. tested
Rabies vaccine, U.S.P., duck embryo[a,b]	0/34
Rabies vaccine, U.S.P., rabbit brain[b,c]	0/34
Rabies vaccine, caprine brain[b,d]	0/24
Rabies vaccine, ERA[e,f]	0/34
Rabies vaccine, Endurall R[f,g]	0/24
Rabies vaccine, Trimmune[b,h]	0/10
Feline distemper vaccine, Delpan[b,i]	0/24
Canine distemper-hepatitis vaccine, CD-H Enduracel[e,f]	0/24
Controls given distilled water	0/22
Controls inoculated with street virus	15/15

[a] Eli Lily and Co., Indianapolis, Ind.
[b] Inactivated virus vaccine.
[c] Semple vaccine, National Drug Co., Philadelphia, Pa.
[d] Bandy Laboratories, Inc., Temple, Tex.
[e] Jensen-Salsbury, Inc., Kansas City, Mo.
[f] Living attenuated virus vaccine.
[g] Norden Laboratories, Lincoln, Neb.
[h] Fort Dodge Laboratories, Fort Dodge, Iowa.
[i] Dellen Laboratories, Inc., Omaha, Neb.

The skins of 208 mice that had been given one of eight vaccines were all negative (Table 4). All of 15 rabies virus-inoculated controls had positive skin results, and all of 22 water-inoculated controls had negative skin results.

DISCUSSION

The results on skin specimens in these studies agreed with and expanded upon previously re-

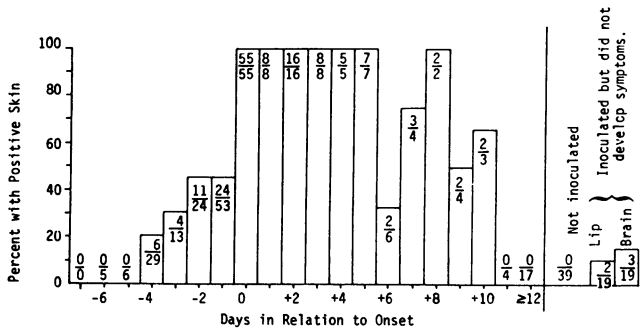

FIG. 2. Results of IF examination of skin temporally related to onset of illness in experimentally infected mice: a composite of three blind-coded experiments with multiple virus isolates and routes of inoculation (number positive/number tested).

ported findings (2). A positive result from examination of skin by IF was closely, but not absolutely, associated with the presence of rabies virus antigen in the brain, also detected by IF. Even in an animal with the earliest detectable symptoms, a positive result obtained by examination of skin had a high degree of correlation with a final diagnosis. Subtle or equivocal results must be interpreted with great care and may be inadvertently subjected to examiner bias by the history of the case. A negative result from IF examination of the skin was strongly associated with a negative result from the brain; Positive brain results in the absence of positive skin results may be the result of sampling in advance of the dissemination of rabies virus from the brain into the peripheral nerves of the face. Also there may be irregular distribution of virus antigen in nerve fibers.

A skin biopsy specimen is actually a small sampling of the entire nerve fiber complex of the face subject to the dissemination of rabies virus antigen. We can merely surmise that the most involved regions and nerve fibers are being sampled, or that the centrifugal dissemination of virus occurs in a uniform manner from one animal and one nerve fiber to the next. In the case of dogs, the lateral sensory papillae on the cheeks (four in most dogs) contain tactile hair follicles and are thought to be optimal for examination. This is because hair follicles are surrounded by a nerve plexus, the size of which is proportional to the size of the follicle (11). Tactile hairs, being very large, are an easy landmark by which to locate large concentrations of nerve fibers, increasing the likelihood that virus will be present in quantity sufficient to be detected. Repeated sampling can exhaust the supply of these, however, so that further sampling must then be from a suboptimal location, less likely to be positive.

Also, the quantity of virus produced in a given infection likely varies from case to case. Occasionally, a small amount of virus seems to be associated with a typical rabies syndrome, so that limited amounts of virus antigen are detected, even in the brain (Table 2). The examination of skin biopsy tissue is accomplished by microscopy, and however sophisticated a procedure is used, it should not necessarily be expected to have the sensitivity of an amplification technique such as mouse or tissue culture propagation, wherein a small quantity of virus is increased. Also, when tests are conducted late in the course of the disease, the presence of antigen may be partly or wholly obscured by the presence of central nervous system-produced antibody, which diffuses into the general circulation in quantity sufficient to combine with available antigen (8). It is essential to use 970× magnification for final readings, as lower magnification greatly compromises the sensitivity of the examination. The variable results obtained by examination of skin tissue of various species of bats seems to reflect the mystery surrounding the interaction between rabies virus and the bat host, but may also reflect anatomical differences (e.g., thin, delicate skin and smaller nerve fibers).

The increasing positivity of skin IF results as the time of onset of illness approached, reinforced the recognized principle (10) that the virus of rabies proceeds from its point of inoculation to the brain and thence centrifugally into peripheral nerves. Thus, a time discrepancy is understandable between detectability of virus in brain and in peripheral nerves of cranial origin (14). These experiments indicated that symptoms of rabies could be predicted, up to 4 days before onset in 20% of mice, and that all sick mice had IF-positive skin. Some of the mice that sickened and recovered had detectable antigen in the skin; others were negative apparently because of inadequate or decreasing amounts of viral antigen present in the nerve fibers. Some lack of fluorescence may also be attributed to blocking of antigen by antibody formed in response to the infection.

The history of the animal involved is paramount in interpretation of results; e.g., a positive skin result in a healthy-appearing animal means either that symptoms will appear in a short time, that the infection is resolving and the antigen is residual, or that a subclinical infection is underway. In any event, the presence of rabies virus antigen in nerve fibers of the skin is a significant finding. Temporal correlation of the presence of antigen in the skin with the presence of infective virus in the saliva is a question that will require further experimentation.

Negative skin biopsy results in species for which adequate data are available (dogs and cats only) have a high degree of reliability, especially when coupled with good history and clinical observation. Second and perhaps third biopsy examination taken after 2 to 6 days, when rabies antigen should have developed to detectable levels, can provide clinically relevant results.

Prior administration of rabies or other virus antigens used in normal immunizations might conceivably result in a residium of antigen, which could be a source of false-positive results obtained by an immunohistological examination. Previous studies on brain tissue of dogs given preexposure immunization (9) and skin of mice given postexposure immunization (13) against rabies have not supported this suspicion. The results of our studies also showed that previous administration of living or inactivated rabies, or other virus vaccines commonly used in dogs or

cats, should not be expected to result in false-positive results. That is, dogs or cats having produced bites and in need of evaluation for their rabies infection status can be reliably examined with no regard to prior immunizations.

The importance of histological as well as immunological evaluation of results of skin biopsy examination also bears emphasis. It should be noted that the anatomical placement and structure of fluorescing antigen is an important consideration. Only antigen occurring in discrete rounded dots sometimes linear in placement, a lack of any angular appearance, and occurring in locations known to contain concentrations of nerve fibers can be considered significant. Fluorescing antigen of this sort can be considered the result of intraneural replication and dissemination from the brain (assuming it to be in terminal fibers of cranial nerves), in other words, a product of the invasive abilities of the virus. It seems reasonable to assume that avirulent virus or antigen would lack such a histological characteristic.

It should also be emphasized that data are inadequate to assess the infection status of nondomestic species (e.g., skunks, foxes, raccoons, etc.) by the microexamination of biopsied skin. It does seem prudent to place reliance upon results obtained by IF examination of properly secured and processed skin biopsy tissue from biting dogs or cats, or as an aid in the early differential diagnosis of disease in other species.

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