Development of Rabies Inhibiting Substance in Skunks Infected with Rabies Virus

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INTRACEREBRAL inoculation of mice with suspensions of suspected tissue has been considered the definitive diagnostic technique for rabies. Earlier workers had recognized that occasionally rabies virus could not be demonstrated by this method from tissues of clinically rabid animals and used the term "auto-sterilization" to describe this phenomenon (*1–5*). Johnson (*6*) summarized these findings: "In dealing with animals, however, isolation of the virus becomes increasingly difficult the longer an animal lives after the onset of the disease, and when death is delayed for a week or more it may be impossible to isolate the virus."

A substance which neutralized rabies virus in the tissues of a fox and of skunks infected with native or "street" virus was originally reported in 1962 (*7*). This substance was later demonstrated from the tissues of a larger series of foxes and skunks and was called rabies inhibiting substance (RIS) (*8*). Bell, in 1964, noted the development of neutralizing antibody in tissues as well as serum of mice that were inoculated intraperitoneally with rabies virus from several sources (*9*).

The following study was designed to ascertain the time when RIS developed in relation to the clinical course of rabies in skunks, the relationship of RIS to serum neutralization (SN) antibody, and the effect of RIS on the amount of virus that could be recovered from certain tissues.

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Materials and Methods

Skunks. Sixteen striped skunks (*Mephitis mephitis*), born in captivity, were used. At the beginning of the study, 11 skunks were approximately 4 months of age and 5 were approximately 16 months of age. Each skunk was penned in an individual cage during the entire study period. The cages were separated so that direct contact between animals was not possible.

Mice. Virus isolation, titration, and neutralization tests were made in 3- to 4-week-old albino mice of the CFW strain. The titration of the infecting inoculum was made in HA/ICR strain mice of the same age.

Virus. The virus used to infect the skunks was a salivary gland isolate from a skunk, which has been described in another report (*10*). Fixed virus (CVS 56) was used in serum neutralization tests, which were performed according to recommendations of the World Health Organization (*11*, *12*). Tissue neutralization tests for determining RIS were performed as described earlier (*7*).

Diluent. All tissues were suspended in 20 percent horse serum in buffered water (pH 7.6 to 7.8) with 2 mg. streptomycin sulfate and 1,000 units of sodium penicillin G per milliliter.

Methods. The skunks were inoculated and the inoculum was titrated on September 26, 1961, at the Midwest Rabies Investigation Station, Poynette, Wis. Specimens were collected

from the inoculated skunks at the same location. Virus, SN antibody, and RIS titrations of the specimens were conducted at the Communicable Disease Center Rabies Investigation Laboratory in Atlanta, Ga.

Mice were inoculated intracerebrally with 0.03 ml. of the test suspensions of skunk tissue and observed daily. The observation period for SN and RIS tests was 14 days, for virus isolation and titrations it was 28 days. Each skunk was inoculated intramuscularly in the right side of the neck with 0.3 ml. of salivary gland suspension in 20 percent normal horse serum. This inoculum contained 20,000 mouse intracerebral $LD_{50's}$ of virus. All animals were observed daily for clinical signs of rabies.

One submaxillary salivary gland was removed surgically from half the skunks either prior to or during the clinical course of the disease. Surgery was performed while the animals were anesthetized with pentobarbital sodium.

Beginning on the 10th day after inoculation and twice a week thereafter, saliva and serum samples were collected from all live animals, except as noted later. Salivation was induced by intramuscular inoculation of pilocarpine nitrate, as described in a previous report (*10*). Blood was drawn by cardiac puncture, and the serum was separated and frozen for storage until SN antibody studies were done.

The skunks were divided into four groups: (*a*) three were killed and necropsied at the onset of clinical signs, characterized by irritation, (*b*) four during the stage of furor, characterized by aggressive behavior, (*c*) three while in the paralytic stage, and (*d*) four after dying of rabies. Two skunks did not develop rabies during the study.

At necropsy the brain, submaxillary salivary glands, a portion of one kidney, one adrenal gland, one testis of each male, and mammary tissue and a portion of the uterus of each female were removed aseptically. Slides were prepared from the brains and salivary glands for fluorescent antibody staining. Brains, salivary glands,

Table 1. Presence of SN antibodies or RIS in skunks, by clinical stage of disease when killed or dead of rabies

Stage when killed or dead of rabies	Number tested	With SN antibodies		With RIS	
		Number	Percent	Number	Percent
Early:					
Onset	3	1	33	0	0
Furor	4	1	25	0	0
Late:					
Paralysis	3	3	100	[1] 2	67
Dead	4	2	50	[1] 2	50
Total	14	7	50	4	29

[1] With SN antibodies also.

NOTE: SN=serum neutralizing; RIS=rabies inhibiting substance.

Table 2. Quantity of rabies virus isolated from brains and salivary glands of skunks

Tissue	Amount of virus isolated (mouse LD_{50})					
	With RIS and SN antibodies		With SN antibodies		With neither RIS nor SN antibodies	
	Mean	Median	Mean	Median	Mean	Median
Brain	3, 380	3, 160	6, 900	10, 000	14, 400	25, 000
Salivary gland	20	0	871	10, 000	95, 400	250, 000

Table 3. Rabies virus and RIS demonstrated in various tissues of 14 skunks (6 males, 8 females) infected with rabies

Tissue	Number with virus	Number with RIS
Brain	14	3
Salivary gland	12	4
Adrenal gland	10	2
Kidney	6	4
Testis	1	0
Uterus	2	2
Mammary gland	1	1

and saliva were homogenized and suspended in the diluent described and immediately frozen at −65°C. for further studies. Other whole tissues were frozen at −65°C. Tissues and serum were packed on dry ice and shipped to the CDC Rabies Investigation Laboratory.

Results

Fourteen of the 16 skunks inoculated died of rabies, as determined by fluorescent antibody staining of brain material. Two animals survived the 68-day observation period. Virus was not isolated from the saliva or tissues of either of these animals, nor were SN antibodies demonstrated from their serum specimens.

Rabies virus was isolated from three of the eight submaxillary salivary glands removed surgically during the observation period; two of the virus-negative salivary glands were from animals that did not develop the disease. Virus was isolated from saliva collected prior to surgery from two of the three skunks from which a virus-positive salivary gland was removed. Virus was not isolated from saliva collected 3 days prior to the removal of a gland of the third skunk, but was isolated from a saliva sample taken the day after surgery. Saliva samples were not taken from any of the animals on the day of surgery.

The longest period that rabies virus was detected in saliva before clinical signs were observed was 7 days in two animals; another had virus in its saliva 6 days before signs of rabies were observed. No rabies virus could be isolated from the saliva of another skunk on the day it was killed, although a large amount of the virus was present in its saliva 4 days earlier; the serum from this animal contained SN antibody 18 days before it was killed and RIS was demonstrated in its brain and salivary gland after death.

SN antibodies were demonstrated in 7 of the 14 animals infected (table 1). Of the seven skunks with SN antibodies, RIS was demonstrated in the tissues of four but not in the tissues of any of the other animals nor in any of the salivary glands removed prior to death

Table 4. Chronicle of observations on 14 skunks infected with rabies

Animal No.	Stage when killed or dead of rabies	Postinoculation day					After death			
		Salivary gland removed	First virus in saliva	First SN antibody	First signs noted	Killed	Virus in salivary gland	RIS in salivary gland	Virus in brain	RIS in brain
407	Onset	[1] 20	17	ND	20	22	+	−	+	−
410	do	NR	17	20	20	20	+	−	+	−
415	do	NR	18	ND	18	18	+	−	+	−
409	Furor	[2] 15	21	ND	28	30	+	−	+	−
411	do	NR	24	ND	24	25	+	−	+	−
413	do	[1] 20	21	ND	21	21	+	−	+	−
416	do	NR	18	16	16	16	+	−	+	−
402	Paralysis	[1] 30	28	28	22	31	+	−	+	−
412	do	NR	18	11	21	29	−	+	+	+
414	do	NR	24	24	26	31	+	+	+	+
403	Dead	[2] 15	ND	ND	23	23	+	−	+	−
405	do	NR	14	17	21	22	+	+	+	−
406	do	NR	ND	ND	19	19	+	−	+	−
408	do	[2] 10	14	14	20	26	−	+	+	+

[1] Virus isolated. [2] Virus not isolated.

NOTE: NR=not removed; ND=never demonstrated; +=present; −=absent.

(table 1). The influences of RIS and SN antibodies on the mean and median rabies virus titers of brains and salivary glands are shown in table 2.

Table 3 indicates the frequency of demonstration of rabies virus and RIS for each of the tissues studied, and table 4 presents the chronicle of observations made on each skunk which developed rabies.

Discussion

Failure to isolate rabies virus from fluorescent antibody positive brains and salivary glands of two skunks and a fox, which developed SN antibodies prior to death from rabies, has been reported (*7*). Several skunks and foxes, dead of rabies, with fluorescent antibody positive brains and salivary glands from which rabies virus could not be isolated were reported in a subsequent study; however, SN antibody determinations were not done (*8*). In both studies a rabies inhibiting substance (RIS) was demonstrated from virus-negative brains and salivary glands.

In the present study, saliva samples collected from three skunks during the clinical course of the disease contained appreciable amounts of rabies virus; however, virus could not be isolated from the salivary glands of two of these animals and only a trace was isolated from the salivary gland of the third after death. Since SN antibodies were demonstrated from all skunks from which RIS was demonstrated, confirming the earlier observation (*7*), a relationship apparently exists between the presence of RIS in tissues and SN antibody.

As shown in table 2, less virus was recovered when SN antibody was present, and even less virus was present when both RIS and SN antibodies were present. This may result in failure to isolate rabies virus by mouse inoculation, although fluorescent antibody staining reveals the presence of rabies antigen in the tissues. Since RIS seems to develop late in the clinical course of the disease, it is possible that an animal might be capable of transmitting the virus during the furious stage of the disease even though virus could not be isolated from the tissues after death.

Conclusions

RIS was shown to develop late in the clinical course of rabies infection. It may produce in vivo neutralization of rabies virus to various degrees. RIS in tissues may interfere with the diagnosis of rabies by mouse inoculation but does not appear to affect the diagnosis of rabies by the fluorescent antibody technique. In skunks and foxes, fluorescent antibody and mouse inoculation tests of rabies may be in disagreement. If either of these tests is positive a diagnosis of rabies is valid.

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GRANTEE INVENTIONS

Determination of Amino Acids in the Presence of Peptides and Proteins

CUPRIC ION will block the reaction of nitrophenyl compounds (as picrylchloride and picrylsulfonate) with dipeptides, polypeptides, and proteins, but will not inhibit the reaction with amino acids (see table).

Readings were made at 420 mu and E calculated for a 1 M solution in a 1 cm. light path; 1 mg. per ml. of picrylsulfonate incubated 30 minutes at 37° C., dipeptide or equivalent amino acids were at a final concentration of 0.25 u M and a final pH 9.5 with borate buffers.

Reaction of amino acids and peptides with picrylsulfonate

Cupric ion molarity u M	Leucine plus glycine $E \times 10^{-4}$	Leucylglycine $E \times 10^{-4}$
0. 00	1. 05	1. 05
. 25	1. 02	. 05
. 50	. 97	. 02
1. 00	. 92	. 01

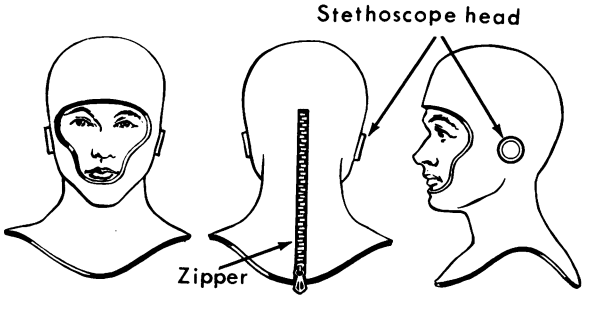
The reaction was used extensively in the assay and study of peptidases and proteases with dipeptides, polypeptides, and proteins as substrates, and for the measurement of free amino acids, bound amino acids, and peptides in biological fluids.

Cupric ion also blocked the reaction of nitrophenyl compounds with dipeptides with both amino acids of the D configuration, but not with dipeptides containing a D and an L amino acid. Thus, the isomers were readily separated either by extraction of the nitrophenyl compounds with organic solvents or by gel filtration with Sephadex or similar materials. The cupric complexes of the dipeptides were of a large molecular weight and were not retarded, whereas the DL and LD dipeptides were retarded.—Prof. Francis Binkley, *Emory University. This technique was developed under Public Health Service grant No. AM-06089.*

Aquatic Auditory Adaptive Device

AN AQUATIC AUDITORY adaptive device to enhance the localization of sound sources in water has been developed. The device should be useful in training divers to localize underwater sound by decreasing the amount of sound energy reaching the inner ear via bone conduction and, at the same time, amplifying some of the sound reaching the meatus. It may help divers maintain a high level of auditory efficiency for long periods because it creates an auditory environment approximating that of air.

The device consists of (*a*) a pressure reduction layer of ½-inch thick foam neoprene covering the cranium, jaw, and neck, and (*b*) flat stethoscope heads mounted over the ears. The diver wears a face mask and uses a scuba mouth piece. It differs from an earlier device intended to reduce the masking effect of bone-conducted sound developed by John M. Ide (Signalling and Homing by Underwater Sound: For Small Craft and Commando Swimmers, NRL Sound Report No. 19, 1944). Ide's device consisted of a ½-inch thick sponge rub-



ber strip, 4 inches wide, running from the forehead to the base of the skull, incorporated in a rubberized canvas helmet which left the ears exposed to the water.

The new device covers the entire head and neck except for the mouth and that portion covered by the face mask. This serves two purposes: (*a*) providing thermal insulation for the diver working in cold water, and (*b*) creating optimal conditions for localization by insulating the bones of the head from much of the sound energy, allowing the ears to operate as independently as they do in air. The pinnae are contained inside the neoprene and access to the sound energy via the meatus is provided by a 3/8-inch hole leading into a flat stethoscope head mounted on the outside of the helmet.—STEPHEN H. FEINSTEIN, *research psychologist, and* JAMES G. CRUMMETT, *senior electronics technician, Stanford Research Institute.* *This invention was developed under Public Health Service grant No. NB–04738.*

Recommendations on Influenza Immunization

The Public Health Service Advisory Committee on Immunization Practices recommends that annual influenza immunization, not currently indicated for all persons, be given to those in groups known to have high mortality rates from epidemic influenza.

Persons of all ages suffering from chronic debilitating diseases comprise these groups. Included are patients with rheumatic heart disease, especially mitral stenosis; cardiovascular disorders, especially frank or incipient cardiac insufficiency; chronic bronchopulmonary diseases; diabetes mellitus; and Addison's disease. Patients in nursing homes, chronic disease hospitals, and the like should be vaccinated since disease can spread easily in such environments once an outbreak begins. Routine immunization is not recommended for pregnant women unless they fall into one of the noted groups.

Vaccination ideally should begin soon after September 1 and be completed by mid-December. Immunization should be completed before influenza occurs in the immediate area since antibodies take 2 weeks to develop.

Persons not vaccinated since July 1963, when the last major change in vaccine formulation was made, should receive an initial subcutaneous dose of polyvalent vaccine followed by a second dose 2 months later. Even a single dose, however, can afford significant protection. A second dose given as early as 2 weeks following the first will enhance protection. Those vaccinated since July 1963 need receive only a single booster of vaccine at the dose level specified for the primary series.

Although recently isolated influenza viruses show minor antigenic alterations of type A2 and B strains, these variations are not of major significance and composition of the 1966–67 vaccine is unchanged from that prepared for 1965–66. Adults and children over 10 years of age should receive 1.0 ml. of vaccine subcutaneously on two occasions; children 6–10 years, 0.5 ml. subcutaneously on two occasions; and children 3 months–5 years, 0.1–0.2 ml. subcutaneously on two occasions separated by 1–2 weeks and followed by a third dose of 0.1–0.2 ml. about 2 months later.