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ADOPTIVE TRANSFER OF IMMUNITY TO RABIES VIRUS

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Adoptive transfer of immunity

to rabies virus

by

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INTRODUCTION

Rabies is a zoonotic viral disease capable of infecting all warm blooded mammals and many birds. Experimentally, the virus has been propagated in reptilian cell culture (Wiktor 1971 and Wiktor and Clark 1972) and transmitted from bats to hamsters with arachnid tissues (Reagan et al. 1955). Viral replication has been demonstrated in neural tissue, muscle tissue and salivary glands. The virus replicates in the cytoplasm of somatic cells and is released by budding, resulting in little cytopathic effect. Incubation periods of the disease are highly variable, ranging from 5 days to several months.

Rabies virus can be shed by way of oral, mammary, and urinary routes. Traditionally, bites by rabid animals have been considered to be the principal means of maintenance and transmission of endemic and epidemic disease. In recent years, however, researchers have demonstrated that viral infection can occur after exposure by ingestion, by aerosol, and by transplacental routes. Viral exposure of mucous membranes, eyes, and open wounds can result in infections. Rabies transmission after a corneal transplant has been reported (CDC 1979a).

Early studies have shown that resistance to rabies is related to levels of specific neutralizing antibody. More recently it has been shown that resistance also can be correlated with the cellular immune response (Wiktor et al. 1972, Mozar et al. 1972 and WHO 1973) and that this cellular response can be adoptively transferred from immunized

donors to naive recipients (Hill 1974 and Tsiang and Lagrange 1980).

It was the purpose of this study to test the possibility of using exogenous lymphocyte extracts and secretions to transfer immunity to rabies virus adoptively in various species of laboratory animals and epidemiologically important wildlife.

Lymphocytes were isolated from either peripheral blood samples or from the spleens of rabies-immunized animals. Lymphokines were collected and partially purified after antigenic stimulation of lymphocyte cultures or after freeze-thaw lysis of the lymphocytes. Processed lymphokines, believed to have transfer factor (TF) activity, were administered to nonimmunized animals which subsequently were challenged with rabies virus. In addition, spleen cell cultures from nonimmune mice were exposed to a processed lymphokine preparation in the presence of rabies antigen as a test for changes in the rates of lymphocyte proliferation.

The efficacy of the TF treatments in vivo ranged from protection, through no effect, to interference with resistance to infection. No effect was noted after exposure of spleen cells from nonimmunized mice to TF in vitro.

LITERATURE REVIEW

Historical aspects

Many pestilences, such as smallpox, plague, typhus, and cholera
that played major roles in the social, economic, and political lives
of people, now can be controlled effectively. Scarlet fever, diphtheria,
and poliomyelitis, which have killed thousands of people, have all but
been eliminated as major health threats in the United States (Burnet
and White 1972). However, rabies, one of the earliest recognized diseases
known to mankind, remains a much dreaded and little understood disease.
Despite the efforts of thousands of researchers throughout the centuries,
it is still prevalent in many parts of the world.

Democritus first described rabies in dogs approximately 500 years
B. C. Aristotle noted the broad host range and zoonotic nature of the
malady when he wrote, in the fourth century, that all animals become
diseased when bitten by dogs suffering from the madness (Steele 1975).
One of the earliest reported cases of sylvatic rabies was that of a
bear in Lyons, France in 900 A. D. (Irvin 1970).

Irvin (1970) suggested that rabies was transported to various
parts of the world by infected dogs carried on the ships of explorers
and merchants, but Constantine (1971) concluded that vampire bats in
Central America were infected with rabies virus prior to the discovery
of America and prior to the widespread transportation of dogs.
Conquering troops and their livestock perished with rabies-like symptoms
following bites by these bats. Natives avoided the disease by washing

bat wounds and cauterizing them with wood embers.

In the United States, rabies in dogs was first recorded in Virginia as early as 1793. By the beginning of the nineteenth century the disease had become well-established in foxes and wolves (Irvin 1970). It is unclear today if rabies moved with the westward migration of the American settlers or if its recognition and description progressed with the movement, but by 1860 it was described as having reached the Mississippi River and, in 1899, it was recognized in California (Sikes 1975).

Morphological description

Rabies is a highly fatal disease caused by rabies virus, which is classified as a member of the Rhabdoviridae (Melnick and McCombs 1966). The virus is an enveloped single-stranded RNA virus with a characteristic bullet-shaped morphology (Davies et al. 1963 and Murphy 1975). Surface projections 6 nm long with a knoblike structure at the distal end are seen on electron micrographs. The projections are not normally seen on the planar end of the particles (Hummeler et al. 1967). Mean dimensions of rabies virus particles are 180 nm x 74 nm, excluding the surface projections (Hummeler et al. 1967 and Sokol et al. 1968).

Factors affecting rabies viral infections

The pathogenicity of rabies virus is poorly understood and unpredictable. Initially the furious form of the disease is characterized by malaise and depression followed by a period of hyperexcitability and tetanic spasms of the muscles controlling breathing and swallowing. Death may occur during this stage, or these symptoms may be followed by

progressive paralysis ultimately leading to death. Paralytic or dumb
rabies, in which the predominant symptom is a general paralysis and
in which no stage of hyperexcitability is noted, is also typical.
The clinical signs of the disease are known to vary with 1) the source
of the virus, 2) the host species, 3) the age and the sex of the
infected animal, 4) the inoculation route and dosage, and 5) the
immunologic state of the host (Dierks 1979).

Isolates of rabies virus obtained from naturally infected animals
are considered to be closely related and are classified in a single
antigenic grouping by nearly all investigators who have studied them
with modern techniques for viral classification (WHO 1973 and Dierks 1979).
Highly specific monoclonal antibodies produced by mouse hybridomas failed
to detect different antigenic specificities in rabies street viruses
isolated from naturally infected animals in many parts of the world but
did identify antigenic differences among various laboratory strains
(Wiktor and Koprowski 1978 and Kaplan and Koprowski 1980).

Although isolates of street virus are indistinguishable anti-
genically, variations have been reported in the characteristics of
the disease produced by rabies viruses isolated from different animal
species. Isolates from spotted skunks (Spilogale putorius) and Arctic
wolves (Canis lupus) are characterized by a long incubation period
in experimental animals and a low pathogenicity in adult mice. In
natural infections of these two animal species the virus is found to
have a tropism for lung and muscle tissue and does not produce the
Negri bodies characteristic of other rabies isolates (Bisseru 1972).

These isolates were not recognized as rabies virus prior to the development of the suckling mouse test or the rabies fluorescent antibody (FA) technique for diagnosis of the disease (Bisseru 1972).

In addition to variations in the characteristics of infection with diverse isolates of virus, the susceptibility of different species of animals to infection with a specific viral isolate is variable. It has been believed, since the time of Pasteur, that isolates of rabies virus propagated in one animal species are more lethal to the homologous species than to an unrelated species. For example, Sikes (1962) determined that foxes were 100 times more susceptible to infection with rabies street virus of fox origin than were skunks. However, Parker and Wilsnack (1966) inoculated 30 foxes and 30 skunks with an isolate from a skunk and found no significant difference between the susceptibility of the two recipient species. They did observe longer incubation periods and higher titers of virus in salivary secretions of skunks as compared to fox salivary secretions. Bats and mustelids are reported to develop subclinical infections with rabies virus but this rarely occurs in dogs (Bisseru 1972).

Sikes (1975) identified rodent and lagomorph species as being either extremely susceptible or highly susceptible to rabies infection. Winkler et al. (1972) inoculated groups of gray squirrels, kangaroo rats, and cactus mice with rabies viral isolates of bat, raccoon, or fox origin and found that gray squirrels were highly susceptible to rabies virus of raccoon origin and much less susceptible to infection with rabies virus of bat origin. Kangaroo rats were highly susceptible to the fox rabies isolate; cactus mice were 20 times more resistant

to infection with the virus inoculum of fox origin than were the kangaroo rats.

Historically, birds have been considered to be rabies-resistant. However, naturally acquired clinical rabies infections in birds have been reported (Schneider and Burtscher 1967 and DuBose 1972). Von Lote (1904) considered chickens and pigeons to have been more resistant to experimental rabies infections than were predatory birds.

Gough and Jorgenson (1976) reported that 23% of predatory birds and 2% of nonpredatory birds tested were seropositive for rabies virus. All nonpredatory seropositive birds, including the European starling (Sternus vulgaris) and the common crow (Corvus brochyrhynchos), were scavaging species. Jorgenson et al. (1976) were able to demonstrate rabies infection in a great horned owl (Bubo virginianus) after viral exposure by the oral route. Seroconversion occurred and virus was reisolated, but no clinical signs of infection were noted.

Younger animals are usually more susceptible to rabies infection than are older ones (Sikes 1975). Personnel at the Center for Disease Control (CDC) suggest that young cats and dogs are more susceptible to the disease and have caused more human exposures to rabies than have mature animals (CDC 1979d). The relatively higher sensitivity seen when using suckling mice in the rabies mouse inoculation test as compared with using weanling mice is well-established (Koprowski 1973a and Bisseru 1972).

The importance of sexual differences in the susceptibility to rabies infection has been investigated. No differences have been seen

in laboratory mice (Koprowski 1973a). Bigler et al. (1973) found no significant difference in infection rates between male and female raccoons in Florida except that a preponderance of infection in adult females was seen during the breeding season. This same phenomenon was reported by Friend (1968) in foxes in New York and by Verts (1967) in skunks in Illinois. Reproductive conditions, such as aggressiveness and physical contact associated with mating and territoriality, may result in an increased incidence of rabies in both sexes (Ulbrich 1969 and Wandeler et al. 1974a, citing Kauker and Zeetl 1960 and 1963). Biting and agressiveness during mating of captive striped skunks is considerable and can result in bleeding wounds for both sexes. Fighting has been observed between males in breeding condition, but fighting was absent when several females in estrus were exposed to a single male (Jorgenson and Gough, unpublished data).

Steele (1973) has suggested that clinical rabies in female bats may be associated with the stress of gestation and parturition. He stated that the greatest stress is associated with lactation and this may result in latent infections becoming active rabies cases.

The bite of a susceptible animal by an infected animal shedding virus in its saliva is the usual means of rabies transmission and bites inflicted on the head, neck, and hands are more likely to develop into fatal infections than are bites to other parts of the body (Sikes 1975). The principal reason for this phenomenon is that the head, neck, and hands are highly enervated and the primary mode of rabies viral spread in the body is along the peripheral nerves to the central nervous system (CNS)

(Dean et al. 1963, Johnson 1965 and Baer et al. 1968). Rabies virus progresses from the site of inoculation to the CNS via the peripheral nerves at a rate of 3 mm per hour (Kaplan 1969). Thus,rabies virus inoculated at the distal portions of the nervous system or where there is a relatively lower supply of nervous tissue is more likely to be inactivated by the host.

Viral transmission by means other than bite by a rabid animal is possible. The transmission of rabies virus by aerosol to humans (Irons et al. 1957 and Winkler et al. 1973) and animals (Constantine 1962 and Fischman and Ward 1968) has been reported. The virus can penetrate the unbroken tissues of the eyes, oral and nasal cavities, and the surfaces of other mucous membranes (Reagan et al. 1955, Robertson and Beauregard 1964, Soave 1966, Fischman 1969 and Correa-Giron et al. 1970). Irvin (1970) has suggested that transmission of the virus by means other than bite is of primary importance in the maintenance of the virus in the endemic sylvatic cycle of the disease.

The immunological state of the host is an important parameter governing the outcome of a rabies viral infection. Natural immunity to rabies infection in animals is documented extensively by Bell (1975). Detectable levels of protective antibody can be induced in a normal host in 7 to 10 days (Atanasiu et al. 1967). If the host's immune system has been exposed previously to rabies antigen, antibody can be detected as early as 3 days after a secondary exposure. A maximum cellular immune response has been reported to occur at 6 to 8 days after primary stimulation and at 3 days as an anamnestic

response (Hill 1974 and Wiktor et al. 1974). Since rabies virus characteristically exhibits a relatively long incubation period, about 1 to 2 months in man (Hattwick 1974), after natural exposure to the virus, the immune system of a normal host can, either by natural or acquired means, usually inactivate the virus before it replicates in the CNS (Janis and Habel 1972).

Finally, the action taken by the host can be an important factor governing the outcome of exposure to rabies virus. The value of wound cleansing by flushing with water and of cauterization has been known since the time of Celsus 100 years A. D. (Parker 1969). Enright et al. (1971) have described the relative values of the use of 65 chemical compounds in preventing rabies in mice. They found that most of the active compounds were solvents or detergents. These compounds were only effective when applied locally near the site of viral injection. Benensen (1970) states that the most effective rabies prevention is the immediate and thorough cleansing of all types of wounds caused by bite or scratch by an animal with rabies or suspected of being rabid. In cases of exposure to a known rabid animal, he further suggests infiltrating the area beneath the wound with rabies hyperimmune serum. This treatment should then be followed with the administration of rabies vaccine or rabies vaccine and hyperimmune serum, according to the recommendation of CDC (Corey and Hattwick 1975 and CDC 1980b) and the World Health Organization (WHO 1973).

Epidemiology

The epidemiology of rabies virus in nature, with the ultimate
goal of its control and eradication, is probably the most reported
facet of the disease. However, due to the difficulty in recognizing
and manipulating the myriad of environmental factors affecting the
transmission and the maintenance of the virus, epidemiology is one of
the least understood aspects of rabies (Irvin 1970).

Rabies virus is pathogenic for all poikilothermic animals (Tierkel
1959 and Kaplan 1969) and experimental infections have been established
in all mammalian species tested (Irvin 1970). Of the 19 orders of
mammals, however, only the Carnivora and the Chiroptera are of importance
as reservoirs of rabies. It is suggested that the Rodentia may be
important in localized areas (Irvin 1970). The disease is seen in the
Artiodactyla, Perissodactyla, and Primates but only as incidental
hosts (Irvin 1970).

Nonmammalian animals such as birds are occasionally reported as
rabid; however, cold-blooded animals are generally thought to be refrac-
tory to the virus. Rabies virus has been reported to persist in the brain
of a tortoise without causing any apparent ill effects (Irvin 1970).
Wiktor and Clark (1972) reported that the virus can replicate in reptilian
cell lines originating from a viper snake, a gecko, a lizard, and a turtle.
Experimental transmission, using insects as vectors, have been largely
unsuccessful (Bell et al. 1957). However, hamsters have been infected
with rabies after rectal instillation of a macerated suspension of

ticks which had fed on rabid bats (Reagan et al. 1955).

In the United States prior to 1960 rabies was most often reported in domestic animals, with dogs being the species most frequently reported rabid. However, with the development of effective rabies vaccines, the establishment of vaccination programs, and the control of stray dogs, the incidence of rabies in dogs has been reduced considerably. In 1953, the year after low egg passage (LEP) chick embryo origin (CEO) vaccine for dogs was licensed for use in the United States, 5,688 cases of dog rabies were reported in the United States; in 1979, only 194 cases were reported. Reported cases of rabies in skunks were 319 and 3,031 in 1953 and 1979, respectively (CDC 1980a). In 1960, for the first time since 1938 when rabies became a reportable disease on a nationwide basis, the number of reported cases of rabies in wildlife surpassed the number of cases reported for domestic animals --1,836 sylvatic cases and 1,619 domestic animal cases (CDC 1977a). Although the change in the relative prevalence of domestic and wildlife rabies, as extrapolated from reporting data, may be a result of an increasing awareness of the disease in the wild animals, Tabel et al. (1974) described a similar change in the relative prevalence of rabies in wild and domestic animals in Canada. They suggest that the change in the prevalence of rabies in the various animal species is not totally a reporting aberration and that wildlife did not play a major role in the perpetuation of early epizootics in Canada.

Wildlife rabies in the United States is manifested primarily in the families Procyonidae (raccoons), Canidae (red and gray foxes),

Vespertilionidae (bats) and Mustelidae (skunks). Animals from these families have accounted for 98% of the 29,932 cases of wildlife rabies reported during the period from 1970 through 1979 (CDC 1980a). For a review of rabies in raccoons, foxes, bats and skunks in the United States refer to Appendix A.

Vaccines and treatments

Although the development of Pasteur's vaccine was a significant advancement in the attempt to control rabies, modifications have followed that have improved the vaccine's safety, potency, and ease of production. Several vaccines currently are available but no one product has been universally accepted as being superior in every respect (Habel 1973a).

Basically there are three major types of rabies vaccines, differing in the method of viral production: nervous tissue origin (NTO), egg embryo origin, and cell culture origin. Habel (1973a) suggested that all known strains of rabies virus have basically similar antigenic composition and there is no need for the use of local strains of virus in vaccines. Wiktor and Koprowski (1978) and Kaplan and Koprowski (1980), however, using monoclonal antibodies, have shown that there are some antigenic differences among various laboratory strains of rabies virus. If significant antigenic differences are found in street virus isolates, it may be necessary to produce rabies vaccines derived from virus isolated from animals in the geographic area where the vaccines are to be used.

Rabbies vaccines of NTO are used widely throughout the world. Such vaccines primarily are produced by propagation of the virus in mice, rabbits, rats, goats, and sheep and are inactivated with phenol (Fuenzalida 1973, Karakujumcan et al. 1973, Lepine 1973, Lepine et al.

1973, Seligmann 1973 and Selimov and Morogova 1973) or ultraviolet light (Gispen 1973 and Habel 1973b). Only murine NTO rabies vaccines are licensed for use in the United States. Nervous tissue origin vaccines have a major disadvantage in that a relatively high rate of nonspecific paralysis is seen as a result of immunologic factors stimulated by contaminating nervous tissue in the vaccine cross-reacting with the host's nervous tissues (Tierkel and Sikes 1967). Abnormalities were seen in electroencephalograms from 14% of the persons treated with NTO vaccines, and neuroparalytic complications occurred in approximately 1 in 600 vaccinates. The fatality rate following neuroparalytic syndromes was 10% to 25% (Applebaum et al. 1953 and Gibbs et al. 1961).

For veterinary use, Habel (1973a) recommended vaccines containing attenuated live virus of CEO. These vaccines are of two major types: the LEP described earlier and the high egg passage (HEP).

Croghan (1970) described the virus for the CEO-LEP vaccine as a "wild strain" of rabies virus (now termed Flury strain) attenuated by 100 serial passages in wet chicks, then serially passed intracerebrally (IC) in 7 day-old chicken embryos until it became lethal in weanling mice only when inoculated IC. For CEO-HEP vaccines further passages were made until the virus was lethal only to suckling mice inoculated IC. Forty to 50 serial passages are necessary for the production of LEP vaccine and 180 passages are used in the production of HEP vaccine (Koprowski 1973b).

The development of CEO rabies vaccine is regarded as a major advance in efforts to control rabies in domestic animals, and, indirectly in human populations. The advantages of this type of vaccine over that

of NTO include a reduction in instances of nonspecific paralysis, a reduction in the amount of contaminating nervous tissue, an increase in the duration of immunity, a reduction of vaccine bulk, and relative ease and safety of production (Croghan 1970).

Until recently CEO-LEP vaccine was considered safe and effective when used for dogs, although it had caused clinical rabies when inoculated into cats (Vaugh and Gerhardt 1961, Dean and Guevin 1963 and Habel 1973a), cattle (Starr 1959 and Habel 1973a), and some other animal species (Tierkel 1963 and Koprowski 1973b). In tests of efficacy it was shown to protect dogs for at least three years (Sikes et al. 1971 and Bisseru 1972). However, the use of the vaccine was discontinued in 1979 as a result of evidence that it was responsible for several cases of vaccine-induced rabies in dogs (CDC 1979c).

Because CEO-LEP vaccine was lethal for cattle, CEO-HEP vaccine was developed. This vaccine was found to be highly effective when used in either cattle or cats, as well as in dogs. Although the CEO rabies vaccines were significant developments in the attempt to control animal rabies, in 1964 Ableseth was able to develop a new strain of rabies vaccine virus termed the SAD strain (Street Alabama Dufferin, formerly identified as the ERA strain). The SAD virus vaccine was found to be significantly more efficacious than were the LEP and HEP vaccines when used to immunize cats, cattle, dogs, horses, goats, and sheep (Abelseth 1967 and CDC 1977b). In addition to being an effective vaccine for domestic animals, Correa-Giron et al. (1970) found that mice could be infected with SAD rabies virus after inoculation of the buccal and lingual mucosa, the

lungs, and the intestines. Furthermore, Black and Lawsen (1970) de-
monstrated that foxes could be vaccinated by the administration of
large doses of attenuated SAD vaccine by stomach tube. It was later
shown that foxes were more readily vaccinated via the buccal cavity than
via the stomach and intestines with this same vaccine (Black and Lawson
1973). Baer et al. (1971) and Debbie et al. (1972) demonstrated that
SAD vaccine administered directly into the oral cavity initiated the
development of serum neutralizing (SN) antibody titers, and foxes, thus
immunized, resisted challenge for at least 8 weeks post-vaccination.

Attempts to find an ideal bait material for administration of the
vaccine to wildlife included such things as vaccine-treated dog biscuits
(Winkler et al. 1975), eggs (Debbie 1973), and sausage baits (Winkler
and Baer 1976).

Although it appeared that the oral vaccination of foxes in the
wild with the SAD strain of rabies vaccine was feasible, safety testing
of the vaccine in nontarget species has resulted in a reevaluation of
its advisability. Winkler et al. (1975) administered the vaccine to
opossums, cotton rats, hamsters, and chickens. One of the 10 cotton
rats died of vaccine-induced rabies when given liquid vaccine directly.
None of the test animals died when the vaccine was incorporated in
the dog biscuits. In safety testing of the use of lyophilized SAD
rabies vaccine placed in plastic pouches, Black and Lawson (1980)
found that laboratory mice could develop active infections with the
virus.

Although the CEO vaccine has greatly reduced the incidence
of rabies in animals, these vaccines are not effective when used to
immunize humans. Duck embryo origin vaccine (DEV), developed by Peck
et al. (1955), was first licensed for use in man in 1957, and, since
that time, it has been the vaccine that has been used almost exclusively
for human post-exposure rabies prophylaxis in the United States (CDC
1976). This vaccine is prepared with a variant of the Pitman-Moore
strain of rabies virus that was adapted to replicate in embryonated
duck eggs. The virus is harvested at the fourteenth egg passage for
use as vaccine (Hoskins 1973). Although it is less antigenic than are
nervous tissue vaccines, it is relatively safe and has led to prophylactic
immunization of persons such as veterinarians, animal control personnel,
and research workers who are in positions where risk of exposure to rabies
is great (Farrar et al. 1964 and Shipley and Jubelt 1968). One death
and 4 neurologic complications were reported in the first 50,000 persons
treated (Rubin et al. 1973). Based on retrospective data from approx-
imately 424,000 persons treated with DEV between 1958 and 1971, Rubin
et al. (1973) estimated the incidence of major neurological complications
attributed to the vaccine to be 3.1 cases per 100,000 persons vaccinated.

Sikes and Larghi (1967) reported that purified neural tissue
vaccine was superior to DEV when compared in mouse potency, guinea pig
challenge, and SN tests. However, comparative statistics from CDC
have shown DEV to be essentially equal to the Semple type (NTO)
rabies vaccines in preventing human rabies in the United States
(CDC 1976).

Although DEV has proven to be as effective and a safer antirabies vaccine than are the NTO vaccines, occasional failures to confer protection have been observed (Anderson et al. 1966, Dehner 1970 and Anon. 1978). In addition, a variety of reactions, including anaphylaxis, local skin reactions, generalized body aches, abdominal pain, nausea, and vomiting, has occurred after its administration (Anderson et al. 1960, Kaiser et al. 1965, Cowdrey 1966, Eldred 1966 and Rubin et al. 1969).

In trying to determine the reasons for the relatively low antigenicity of DEV, Kuwahara and Burgoyne (1978) found that commercially prepared DEV contained the enzyme neuraminidase which degraded viral antigens. In addition, DEV was found to induce production of neuraminidase by the host. Two cell culture vaccines (human diploid cell vaccine and primary hamster kidney vaccine) have not been found to either contain or induce neuraminidase activity.

A recent advance in human antirabies treatment was the development of techniques for propagating rabies virus in human diploid cell culture. With this system, problems associated with immunological responses to contaminating nervous tissue and contaminating avian protein are circumvented (Prozesky 1978). Wiktor et al. (1973b) were the first to report the results of vaccine trials using human diploid cell vaccine (WI-38) in humans. Their WI-38 vaccine, inactivated with tributyl phosphate, was administered to 8 persons. Five of the 8 vaccinates showed a SN response after 1 inoculation, and 7 of the 8 had a response after 2 inoculations. Serum neutralizing titers after 2 inoculations

with this vaccine were essentially equal to titers generally reached after 14 doses of DEV. A high anamnestic response was found in persons previously inoculated with DEV.

Hafkin et al. (1978), in comparing the WI-38 vaccine with DEV in rabies pre-exposure prophylaxis administered WI-38 vaccine to 19 veterinary students and DEV to 17 others and reported WI-38 to be clearly superior to DEV in its ability to induce SN antibody. They found that 3 doses of the WI-38 vaccine were necessary to induce an adequate rabies antibody titer in 100% of the recipients. Adverse reactions were reported to be essentially the same with both vaccines.

Human diploid cell rabies vaccines (HDCV) currently are being produced by Merieux Institute in France, Behringwerke in Germany, and Wyeth Laboratories in the United States (CDC 1978a). The French and German vaccines have been used to treat more than 100,000 persons in Europe. The Merieux HDCV has also been licensed for use in the United States and has largely replaced the DEV vaccine for rabies post-exposure and pre-exposure prophylaxis (CDC 1980 d and 1981).

In a serologic study of 19 persons to determine the active anti-body response after administration of HDCV alone or in conjunction with rabies immune globulin (RIG), Kuwert et al. (1979) found that no interference of active immunization occured due to the RIG. Instead, an additive effect was seen between passive antibody and actively produced antibody. This is especially significant because RIG has been shown to suppress the antibody response to other rabies vaccines (Loofbourow et al. 1971 and Hattwick 1974). Wiktor and his co-workers found that the

administration of 19S and 7S antibody exerted similar effects (Wiktor
et al. 1971). This inhibitory effect can be partially overcome if
the number of vaccine doses is increased, if vaccination is started
15 days after administration of serum, or if immune serum is given
12-24 hours after vaccination (Wiktor et al. 1971). When hyperimmune
serum and vaccine are administered for rabies post-exposure treatment,
21 doses of vaccine plus booster doses on the 10th and 20th day after
the completion of the initial course of treatment are recommended
(Corey and Hattwick 1975 and CDC 1980a). When HDCV is used for post-
exposure treatment in conjunction with RIG, no additional booster
vaccination is necessary (CDC 1980 e).

Nonspecific resistance to rabies

In further efforts to improve rabies vaccines and treatment regimes,
Baer and Cleary (1972) found that highly concentrated baby hamster kidney
(BHK) vaccine markedly reduced mortality from rabies because of its
ability to induce the in vitro production of interferon (IF). In 1977,
Baer et al. reported that the addition of IF or an IF inducer to an in-
effective rabies vaccine resulted in a marked increase in protection.
Mice receiving rabies vaccine containing the inducer polyriboinosinic-
polyribocytidylic acid (poly I-C) and poly-L Lysine with carboxymethyl-
cellulose (poly ICLC) were more resistant to infection than were mice
treated with vaccine alone. All mice infected with street rabies virus
(0.03 ml in the footpad) and treated 24 hr later with HDCV in conjunction
with poly ICLC were protected from rabies. Mice treated with HDCV alone

had a significantly higher mortality rate (43.8%). This was essentially equal to the mortality rate observed in untreated control mice (49%).

The effect of the IF inducer poly I-C in reducing the lethality of both street virus and fixed strains of rabies virus was evaluated in mice and in rabbits by Janis and Habel (1972). They found that 6 daily intravenous (IV) treatments with 1.0 μg or 0.5 μg of poly I-C, beginning 3 to 24 hr before challenge, protected rabbits from fixed strains of the virus. Three daily intramuscular (IM) treatments with 0.5 μg, beginning 3 hr post-viral challenge, protected rabbits inoculated with street rabies virus. Mice infected with street virus were protected with a single dose of 1.3 μg of poly I-C given either IM or IP 3 hr post-viral challenge. Two or more IM treatments given at the same site as the viral inoculum were found to be more effective than intraperitoneal (IP) inoculations. Protection was seen in mice treated as long as 67 hr after injection with street rabies virus.

Although Janis and Habel (1972) and Baer et al. (1977) were able to demonstrate protection from rabies infection in mice and rabbits treated with IF inducers, other investigators have reported that the IF inducing capacity of poly I-C varies with the host species (Young 1971 and Turner 1972). Atanasiu and co-workers were able to induce high levels of endogenous IF in mice, hamsters and rabbits by IV or IP inoculation of inactivated Newcastle disease virus. With subsequent IM challenge with control virus strain (CVS) rabies virus they found that the rabbits and hamsters were resistant to infection but no protection was observed in the mice (Atanasiu et al. 1970).

Postic and Fenje (1971) reported similar results and stated that for unknown reasons, IF does not penetrate the CNS of mice.

Young (1971) found that poly I-C does not induce high levels of IF in man and, when it is administered at recommended levels as extrapolated from most animal studies, it would be toxic. However, Fenje and Postic (1971) state that the dosage level of poly I-C effective in protecting rabbits from rabies in their experiments is within the range of doses contemplated for antitumor therapeutic trials in humans. They have published results of research in which a single dose of 0.04 mg of poly I-C inoculated IV or IM provided protection to rabbits inoculated IM, with 5, 25, and 125 rabbit LD50 of rabies street virus (fox origin).

Exogenous IF has been used as a post-exposure treatment of animals for rabies (Postic and Fenje 1971 and Ho et al. 1974). However, Ho et al. stated that at least 70 million units of IF would be needed for one treatment in a human adult male exposed to rabies. In addition, there is a very short time period after exposure during which TF is effective. Recent progress in the development of methods of TF production in bacterial or yeast cultures may make the use of exogenous IF in the treatment of human rabies feasible.

Other studies of nonspecific resistance to rabies viral infections in mice, rats and guinea pigs have been reported. Haldar et al. (1977) treated mice (3 mg), rats (15 mg), and guinea pigs (30 mg) with powdered Mycobacterium phlei suspended in veronal buffer. A second

group of animals was vaccinated with inactivated rabbit brain rabies
vaccine. The animals were subsequently challenged at 10, 20 or 30 days
post-treatment by IM inoculation with rabies virus (CVS). Similar
resistance to rabies challenge was seen in the 2 groups. No explanation
of the observed results was given. The non-specific resistance exhibited
was attributed to "some other factors."

Banic (1975) reported reduced mortality to rabies infection in
guinea pigs after 7 days of vitamin C treatments (100 mg/Kg) initiated
6 hr after inoculation with a 10% rabbit brain suspension of an un-
identified fixed virus. Nonspecific environmental factors such as
high ambient temperatures have also been shown to retard replication
of rabies virus (Clark and Wiktor 1972 and Bell and Moore 1974).

Specific resistance to infection with rabies virus

Information concerning the immunologic response to rabies virus
has been obtained primarily as a result of vaccine trials. It has been
shown that protection against rabies virus correlated well with the
concentration of specific serum antibody. The passive administration
of immune serum has been shown to help prevent clinical rabies.

The protein moieties of rabies virus have been described (Sokol
et al. 1971, Wiktor 1971, Madore and England 1977 and Zaides et al.
1979). These proteins — the M protein of the nucleocapsid, the EP2
and EP3 structural proteins, and the G protein of the surface projections —
do not all elicit production of the same activities of antibody. The
G protein has been shown to be responsible for eliciting neutralizing

antibody (Wiktor 1971 and Wiktor et al. 1973a). Rubin et al. (1971)
and Dierks et al. (1974) identified IgM with in vitro neutralizing
activity in blood collected from day 10 through day 27 from 39 persons
treated with a full course of DEV. However, antibody of the IgG class
is the only antibody considered to offer significant protection in vivo
against rabies infection (Wiktor et al. 1968, Wiktor et al. 1973a,
Coe and Bell 1977, Grandien 1977 and Turner 1978). Antibody of the
IgM class has a higher valence for viral attachment but it is restricted
to the circulatory system and can not neutralize virus in the tissues.
Turner (1978) suggested that it might neutralize any extracellular virus
in contact with blood in bite wounds but stated that this is very
unlikely.

No work has been published regarding the presence of secretory
IgA response to rabies infection or the capacity of IgA to neutralize
rabies virus. Using the passive hemagglutination PHA test (Gough and
Dierks 1971) Jorgenson and Gough (unpublished data: Veterinary Medical
Research Institute, Iowa State University) were able to detect antibody
in oral secretions from 11 of 15 animals of 6 wildlife species inoculated
IM with either rabies street (skunk origin) or fixed (SAD) virus. One
of 2 raccoons (procyon lotor), 2 opossums (Didelphis marsupialis), 1
of 1 spotted skunk (Spilogale putorius) and 4 of 4 coyotes (Canis latrans)
had antibody in salivary secretions at titers ranging from 1:2 to 1:256.
No conclusion was made as to the class of antibody detected.

Kubes and Gallis (1944) described a phenomenon whereby brain
tissue from rabid animals, vaccinated animals, or animals recovered

from rabies infection was able to neutralize the virus. Bell et al. (1966) speculated that the brain neutralizing substance was locally produced antibody. Later Gough et al. (1974) characterized this brain associated neutralizing substance as immunoglobulin G (IgG). This was later confirmed by Coe and Bell (1977) and Bell and Moore (1979), who further demonstrated that the antibody originated in the CNS.

Several serologic techniques are available for the routine detection of rabies antibody. It is suggested that various forms of the virus neutralization test measure antibody of the IgG class directed against the G protein of the virus and do not readily quantitate antibody of the IgM class (Grandien 1977). The indirect immunofluorescence procedure mainly detects antibody directed against nucleocapsid antigen, as does the complement fixation test (Wiktor 1971, Wiktor et al. 1973a and Schneider et al. 1973). The hemagglutination inhibition and PHA techniques probably measure antibody directed at surface antigens (Grandien 1977).

In an effort to evaluate the immune mechanisms responsible for abortive rabies infections, Wiktor et al. (1972) suppressed the antibody response in 12 mice by irradiation. These mice and 12 nonirradiated control mice were then inoculated IC with HEP rabies vaccine followed 2 hr later by intraplantar inoculation with a lethal dose of street rabies virus of bat origin. All vaccinated animals survived but challenged mice that had not been vaccinated died of rabies infection.

This trial suggested that mechanisms other than either circulating or CNS-associated rabies antibody mediated the protection seen after IC vaccination with HEP. Wiktor's group theorized that survival of the irradiated, vaccinated mice was mediated through IF. Although IF possibly contributed to the protection, the role of cell-mediated immune (CMI) responses must also be considered.

Mozar et al. (1973) reported the development of a strong CMI response in man after vaccination with DEV following exposure to a rabid cat. In that same year the World Expert Committee On Rabies (WHO 1973) suggested that the stimulation of CMI might be crucial for protection from rabies infection.

To further investigate the role of cellular immunity in rabies viral infections, Turner (1973) passively transferred spleen cells from immune mice to naive syngeneic mice, and, on subsequent challenge with virus, no protection was demonstrated in the recipient animals. Shope et al. (1979) reported on the work of Prabhackar who demonstrated that immunosuppressed mice inoculated with HEP rabies virus died, but if lymphocytes from immune syngeneic mice were given at 4 to 11 days post-infection, the mortality was reduced from 90% to 37%.

Using immune rabbit spleen cells, Wiktor et al. (1974) were able to demonstrate an in vitro CMI response to rabies virus. Maximum stimulation occurred 8 days after immunization and the immunity could be detected with cells collected as long as 175 days post-vaccination. They reported that purified virions, viral glycoprotein, and "soluble antigen" were capable of stimulating transformation of blast cells

sensitized with rabies virus, while nucleocapsid was not.

Hill (1974) showed that a cellular immune response in mice appeared as early as 3 days post-inoculation and a maximal cellular response occurred at 6 days. He reported a loss of cellular response by day 20, but a rapid increase in activity was noted after reinfection at 30 days. Live virus was more effective as a primary inducer but the secondary response was stimulated equally by live and inactivated virus. These findings are similar to those reported by Wiktor et al. (1974 and 1977a).

Wiktor et al. (1977a) demonstrated that β-propiolactone-inactivated SAD strain rabies virus inoculated IP into mice generated a strong cytotoxic T-cell response in spleen cells, with a maximum response 6 days post-inoculation. The cytotoxic T-cell response seen in vitro was abrogated by exposing the culture of lymphocytes to anti-thymus antibody and complement prior to the addition of antigen. With 100 plaque forming units (PFU) of live attenuated HEP and SAD rabies virus inoculated IC into mice, maximum cytotoxic responses were seen on day 7 (Wiktor et al. 1977b). No cytotoxic response was generated in mice inoculated IC with 1000 LD50 of street rabies virus of skunk origin. Mice inoculated with street virus developed fatal infections, which Wiktor and his co-workers suggested was a result of the defective operation of the CMI protective mechanisms.

Possible explanations for the failure of street virus to generate a CMI response suggested by Wiktor et al. (1977b) are that the immune system was not sufficiently exposed to the virus or that street virus does not cause antigenic changes on infected cell surfaces that would

be recognized by cytotoxic T-cells. Using the footpad test, Tsiang and Lagrange (1980) have since reported in vivo evidence of a CMI response in mice to rabies virus of fox salivary gland origin. It was necessary to use the Pasteur strain of Mycobacterium bovis (BCG) as adjuvant and 25 to 50 Hemagglutination units (HAU) as compared with 5 HAU of the stimulation inoculum for stimulation in mice sensitized with fixed virus. Using street virus, a peak response was seen on day 4 as compared with 6 to 8 days when using fixed virus.

In an attempt to demonstrate a definite correlation between CMI and the resistance acquired after immunization against rabies, Turner (1979) treated mice with cyclophosphamide monohydrate (Cy) at various time periods prior to immunization with HDCV. The mice were subsequently challenged (IM) with 5 to 10 mouse LD50 of CVS rabies virus. Cyclophosphamide is an immunosuppressant with longer lasting effects on B lymphocytes and antibody formation than on T lymphocytes and CMI responses. He found that protection was inadequate unless immunization with rabies vaccine was delayed until recovery of B-cell function and that CMI responses alone can not provide adequate protection against rabies infection.

Miller et al. (1978) approached the problem of elucidating the relative roles of the humoral and CMI immune responses to rabies virus by selective immunosuppression. Suppression of the humoral response was accomplished with the use of antibody against the IgM isotype (anti-μ- antiserum). Anti-μ-treated mice failed to produce antibody to rabies virus although their T-cell function remained normal as demonstrated by the spleen cell proliferative response to Con-A.

Suppression of cellular responses was accomplished by inoculation of
thymectomized and irradiated mice with syngeneic bone marrow cells that
had been treated with rabbit-anti-mouse thymocyte serum. CMI depression
was also accomplished with the use of Cy. Both of these procedures,
unfortunately, impair activities of B lymphocytes as well as those
of T lymphocytes.

Immunosuppressed and control mice were inoculated IC with a
standard dose (10,000 suckling mouse LD50) of HEP rabies vaccine which
causes subclinical infection in adult mice. Suppression of the
humoral response resulted in a 50% higher mortality rate than was seen
with immunocompetent control mice. A 70% higher mortality rate occurred
with T cell-depleted mice than with immunocompetent control mice.
Slightly more mice died of rabies when treated with Cy as compared with
the bone marrow reconstituted mice. This would be expected because Cy
is known to prevent formation of both B and T lymphocytes.

Miller et al. (1978) concluded that both B and T lymphocytes
are intimately involved in controlling rabies viral encephalitis.
Antibody may be involved in viral neutralization by coating critical
receptors, in complement-mediated cytolysis and virolysis, and in antibody-
dependent cell-mediated cytotoxicity. Finally, antibody-initiated
activation of complement could play a role by causing an inflammatory
response to occur in rabies infected tissues. They suggested that
helper T-cells were necessary for the production of SN IgG antibody.
No conclusions were made as to the role of effector T-cells but the
increased mortality following depletion of T lymphocytes relative to

that occurring after depletion of B lymphocytes implies that CMI also contributes to recovery from rabies. Wiktor et al. (1977b) have stated that the level of effector T-cell function is directly correlated with survival following rabies viral infection.

Although there is considerable controversy as to the exact role CMI responses play in protection from rabies infection, evidence has been presented to establish that both humoral and cellular responses are inseparably involved in the protection observed.

Transfer factor

Cells were first shown to mediate CMI when Landsteiner and Chase (1942), using viable lymphoid cells, transferred reactivity against tubuculin from sensitive to normal guinea pigs. Peripheral blood leucocytes (94-98% pure lymphocyte suspensions), but not granulocytes or other glass-adherent cells, are sufficient to transfer sensitivity (Slavin and Garvin 1964). In 1955 Lawrence observed that leucocyte extracts were as effective as viable cells in the transfer of CMI· He termed the active factor(s) "transfer factor" (TF).

Transfer factor, as originally defined by Lawrence (1955), is the material or materials present in viable cells or extracts of immune lymphocytes responsible for the transfer of CMI to nonimmune recipients. Lawrence (1974) described TF as "a shunt that bypasses the stage of active immunization and converts naive lymphocytes to an antigen-responsive state." The activity of TF has not been found to be limited by species barriers, as shown by in vivo and in vitro studies

(Table 1). Although TF has not been isolated in sufficiently pure form to be fully characterized, a number of physicochemical properties and functional properties have been ascribed to it (Tables 2 and 3).

Adoptive transfer of cellular immunity in humans has been accomplished using various types of preparations containing or derived from circulating blood leucocytes: viable leucocyte concentrates (Lawrence 1949 and 1952), leucocyte extracts prepared by lysis with distilled water or by freezing and thawing (Lawrence 1954 and Lawrence et al. 1960), enzyme-treated (DNase, RNase, DNase+trypsin) leucocyte extracts (Lawrence 1955 and 1959), and antigen-liberated TF (Graybill et al. 1973). Lawrence and Al-Askari (1971) reported that all of these techniques have been applied with equal effects.

Transfer factor(s) have been used extensively in the treatment of various disease states in man. Since it was first used in 1970 (Levin et al. 1970), bacterial (Anttila et al. 1977, Rubenstein et al. 1977 and Hastings and Job 1978), viral (Drew et al. 1973, Kohler et al. 1974, Kackell et al. 1975, Heim et al. 1976 and Mazaheri et al. 1977), fungal (Graybill et al. 1973 and Smith et al. 1976), and parasitic (Liburd et al. 1972) diseases, as well as neoplasms, have been treated with TF with varying degrees of success (Krown et al. 1974, Oettgen et al. 1974 and Byers et al. 1976). Transfer factor has had its greatest application when used to treat certain cellular immunodeficiency states (Goldblum et al. 1973, Levin et al. 1973 and Ammann et al. 1974).

The TF phenomenon was originally described in man and was not observed in other animal systems until 1972 (Burger et al. 1972).

Table 1. Transfer of delayed-type hypersensitivity between
         different animal species

Species	Source
Human to Monkey	Maddison et al. 1972, Gallin and Kirkpatrick 1974, Kirkpatrick and Gallin 1974 and Maddison 1975
Human to Mouse	Adler et al. 1970, Palmer and Smith 1974, Arala-Chaves et al. 1979, Brummer et al. 1979 and Schindler and Baram 1979
Human to Guinea pig	Lesourd et al. 1979 and Mischak and Spitler 1979
Human to Rat	Arrenbrecht et al. 1979
Human to Dog	Tomar and John 1980
Mouse to Human	Vich et al. 1979
Rat to Human	Boucheix et al. 1977
Bovine to Human	Burger et al. 1979, Jeter et al. 1979 and Newell et al. 1979
Bovine to Monkey	Klesius et al. 1975
Bovine to Mouse	Klesius et al. 1978 and Klesius 1979
Bovine to Guinea pig	Lesourd et al. 1979
Bovine to Rabbit	Klesius et al. 1975
Bovine to Dog	Klesius et al. 1975
Guinea pig to Mouse	Potter et al. 1974

Table 2. Immunobiologic properties of TF

Property	Source
Endows recipient with specific sensitivity of donor	Lawrence 1955
Extracts or cell-free supernatants as effective for transfer as viable cells	Lawrence 1955
Function of T cells	Slavin and Garvin 1964 and Hattler and Amos 1965
Nonimmunoglobulin	Baram et al. 1966
Nonantigenic	Rapaport et al. 1965
Interacts with but is not neutralized by antigen	Lawrence and Pappehheimer 1956
Activity not species specific	Adler et al. 1970
Not active sensitization (early onset: 4-6 hr)	Lawrence and Pappenheimer 1956
Not passive sensitization (long duration: years)	Rapaport et al. 1960
Serial transfer	Lawrence 1955
Interferon induction	Emodi et al. 1973
Does not stimulate antibody production	Pappenheimer 1956 and Good et al. 1957

Table 3. Physiocochemical characteristics of TF

Characteristic	Source
Resistant to lysis by distilled water	Lawrence 1955
Dialysable	Lawrence 1955
Lyophilized dialysate stable for as long as 5 yrs. at 4 C	Lawrence 1969
Inactivated at 56 C for 30 min; however Spitler et al. (1973) found TF resists 56 C for 2 hr	Lawrence and Zweiman 1968
Resistant to the following enzyme activities:	

Pronase
    However, Burger et al. (1979)
    found TF to be sensitive to pronase.
	Spitler et al. 1973

DNase	Lawrence 1959
Pancreatic RNase	Lawrence et al. 1960
T 1 RNase	Potter et al. 1974
Trypsin	Lawrence 1959
Destroyed by RNase III	Potter et al. 1974
Positive biuret reaction	Lawrence 1969
Positive Lowry reaction	Kirkpatrick and Gallin 1974
Positive orcinol reaction	Baram and Mosko 1965
Polypeptide: oligopeptide ratio, 2:1	Gottlieb et al. 1973
Contains approximately 12 amino acids and 3-4 RNA bases	Gottlieb et al. 1973
Molecular Wt. 2000-4000	Gottlieb et al. 1973
Density $1.47/g/cm^3$	Gottlieb et al. 1973
Net negative charge	Potter et al. 1974
Isoelectric pnt. pI=1.6	Vandenbark et al. 1977

It has since been described in subhuman primates (Maddison et al. 1972 and Gallin and Kirkpatrick 1974), rats (Liburd et al. 1972), guinea pigs (Burger et al. 1972 and Rosenfeld and Dressler 1974), mice (Palmer and Smith 1974), cattle (Klesius et al. 1975 and Klesius and Fudenberg 1977) and dogs (Thilsted and Shifrine 1979).

The administration of TF by IM and IV routes was found to be equally effective when used to immunize guinea pigs against tuberculin (Lawrence 1974). Successful transfer of tuberculin sensitivity with oral administration of TF has been described (Jeter et al. 1977 and Jeter et al. 1979). Systemic versus local transfer of sensitivity is reported to be dose dependent (Lawrence and Zweiman 1968).

Transfer factor is thought to function as an informational molecule, causing or uncovering antigen-specific receptors on T-lymphocytes. Theoretically, TF may serve as a derepressor of potentially antigen-responsive lymphocytes or as a TF-antigen complex of increased immunogenicity ("superantigen"). The "superantigen" hypothesis is not widely accepted because development of immunity in naive animals subsequent to treatment with TF occurs too rapidly to be explained as an active response elicited by exposure to antigen (Potter et al. 1974). Data obtained from equilibrium dialysis of specific TF against specific antisera (Baram and Mosko 1965) and the absence of an anamnestic antibody response in rhesus monkeys treated with specific TF and subsequently challenged with specific antigen (Maddison et al. 1972) further refute this theory.

In addressing the derepressor hypothesis of Lawrence (1969), Potter et al. (1974) state that, although TF is too small to code for

specific proteins involved in the immune response, it could function as a regulatory element in genetically competent leucocytes. According to this theory, the double-stranded RNA moiety of TF would compete with a cellular DNA sequence for a specific repressor. The repressor, theoretically, would have a greater affinity for the TF molecule than for cellular DNA, and specific gene(s) necessary for the production of proteins (receptors and/or lymphokines) involved in the immune response would be derepressed. Based on the projected theories of size, chemical composition, and steric organization of TF, Potter et al. (1974) speculated that 4^{30} different TF molecules could possibly exist to account for the specificities observed.

Although TF has been used either experimentally or clinically in man for the last 25 years, reports of adverse effects of its use are limited. In studies describing severe adverse reactions in recipients, no conclusive evidence to attribute these to the administration of TF has been presented (Maddison, 1975). Some potential hazards that have been proposed include: contamination of TF preparations through careless handling; stimulation of autoimmunity in the event that the donor may have acquired hypersensitivity to histocompatibility antigens of the recipient; stimulation of an overwhelming hypersensitivity in recipients who are anergic to the organisms causing their disseminated infections or to large tumor masses; and the transfer of unwanted hypersensitivities, such as poison ivy (Maddison 1975 and Basten et al. 1975).

MATERIALS AND METHODS

Experimental Animals

Mice

All outbred mice used in this study were from a colony of
Swiss-Webster mice which has been maintained at the Veterinary Medical
Research Institute since 1972. Mice used for virus isolation and
virus titration procedures were 18-26 days old when inoculated.
Mice used for adoptive immunization procedures were 28-35 days old.

Inbred BALB/c breeding stock mice were purchased commercially.[1]
The 5 to 8-week-old mice used as a source of spleen cells for TF and
for in vitro lymphocyte proliferation assays were raised at the
Veterinary Medical Research Institute.

Rats

Sprague-Dawley descendent outbred rats of mixed sex were purchased
commercially.[2] All rats weighed approximately 275 gm at the time of
inoculation.

Guinea pigs

Guinea pigs were obtained from a random-bred colony maintained
at the Veterinary Medical Research Institute. Guinea pigs weighed

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[1]M. A. Bioproducts, Walkersville, Maryland.
[2]Bio-Lab Corporation, St. Paul, Minnesota.

from 300 to 400 gm when utilized.

Hamsters

Syrian hamsters were obtained from a random-bred colony maintained at the Veterinary Medical Research Institute. Adult hamsters were used for the production of immune lymphocytes in adoptive immunization trials.

Raccoons

Raccoons (Procyon lotor) were collected in Story County, Iowa with the use of boxtraps.[1]

Striped skunks

Adult skunks were captured in north central Iowa in box traps by employees of the Iowa Conservation Commission.[2] These animals were surgically descented and maintained in captivity for approximately four months prior to being used for evaluation of adoptive immunization.

Swine

Adult swine of mixed breeds were used as a source of peripheral blood leucocytes for the indirect leucocyte migration inhibition assay.

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[1]Tomahawk Trap Co., Tomahawk, Wisconsin.

[2]Ronald Andrews, Iowa Conservation Commission, Clear Lake, Iowa.

Viruses

Immunization virus

Three sources of rabies virus were used for immunization of animals
for the production of rabies immune lymphocytes: 1) High egg passage
rabies virus vaccine of chicken embryo origin was purchased commercially[1]
This lyophilized modified live virus (MLV) was reconstituted in sterile
distilled water according to the manufacturer's directions. 2) CVS-27
rabies virus was purchased commercially[2] and propagated in mice. The
lyophilized virus material was reconstituted in M-199 culture medium
and inoculated IM into the right hind leg (RHL). When moribund, the
mice were killed; their brains were removed and stored at -70 C until
used. 3) Dried, killed rabies virus of duck embryo origin purchased
commercially[3] was used in experiments to compare the efficacy of
adoptive immunization alone, vaccine alone, and enhancement of vaccine
by adoptive immunization in mice. A single dose vial of commercial
vaccine was reconstituted in 9 ml of sterile distilled water and '
dispensed into a 10 ml vaccine vial. The vaccine was prepared on the
same day it was to be used.

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[1]Norden Laboratories Incorporated, Lincoln, Nebraska.

[2]American Type Culture Collection, Rockville, Maryland.

[3]Eli Lilly and Company, Indianapolis, Indiana.

In vivo challenge virus

Lyophilized CVS-27 virus was reconstituted in sterile distilled water and inoculated (IM) into laboratory mice (0.05 ml). The animals were killed when they were moribund, the brain was removed and a 20% (W/V) brain tissue suspension was prepared in Medium-199 (M-199) containing 100 units of penicillin, 50 µg of kanamycin and 20% fetal bovine serum. The suspension was centrifuged at 200 x g to remove cellular debris. The supernatant was removed, aliquanted into 10 ml vials and stored at -70 C until used.

In vitro lymphocyte stimulation virus

Stimulation antigen (SAD virus)[2] was propagated in baby hamster kidney-21 (BHK-21) cell culture to a titer of $10^{7.5}$ mouse ICLD50 and stored at -70 C until purified or used for in vitro production of lymphokine.

Virus Purification

Rabies virus lymphocyte stimulating antigen was prepared using the aluminum phosphate precipitation procedure used in the lymphocyte proliferation assay described by Schneider (1973). The purified SAD strain of rabies virus used in the lymphocyte proliferation tests

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[1]International Scientific Inc., Gary, Illinois.
[2]Dr. George Baer, Center for Disease Control, Atlanta, Georgia.

was diluted to $1:10^3$ for optimal stimulation of sensitized spleen cells.

Animal Inoculation-Sensitization with Rabies Virus

High egg passage modified live virus vaccine of chicken embryo origin was administered to guinea pigs and mice by IM injection. The animals were given 2 inoculations 1 week apart. Guinea pigs received 0.5 ml and mice received 0.05 ml of the inoculum per inoculation. Ten to 12 days after the last inoculation the animals were bled and peripheral lymphocytes were isolated for in vitro antigen stimulative lymphokine production.

Duck embryo origin rabies virus was reconstituted in 1 ml of sterile distilled water and homogenized 1 to 1 with either Freund's complete or Freund's incomplete adjuvant.[1] Adult BALB/c mice were inoculated subcutaneously in each of the hind foot pads with 0.01 ml of the vaccine in Freund's complete adjuvant once each week for 3 weeks. A booster inoculation with the vaccine incorporated in Freund's incomplete adjuvant was administered 1 month after the last of the 3 initial inoculations. Control mice were inoculated with Freund's adjuvant diluted 1 to 1 with sterile distilled water. Spleen cells were collected 1 week after the booster inoculation was given and used for the preparation of dialyzable TF or for the lymphocyte proliferation assay.

A suspension of mouse brain containing live rabies CVS-27 was used in the production of virus-stimulated lymphocytes. The suspension

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[1]Bio-Lab Corporation, St. Paul, Minnesota.

was inoculated IM into the RHL of mice (0.05 ml), rats (0.25 ml), hamsters (0.1 ml) and guinea pigs (0.2 ml). When moribund, the animals were bled and peripheral lymphocytes were isolated for in vitro release of lymphokine.

An adult male striped skunk which had been raised in captivity was fed a diet of commercial dry dog food and was provided with a 3.63% solution of levamisol hydrochloride in the drinking water for 30 days. The animal then was inoculated IM twice with β-propiolactone inactivated CVS-27 rabies virus ($10^{6.8}$ MICLD50) in Freund's complete adjuvant in the RHL at 1 week intervals. Seven days after the second immunization, a blood sample was collected by cardiac puncture into a siliconized blood collection tube containing 1.5 mg ethyl-enediaminetetraacetic acid (EDTA)/ml blood. The lymphocytes were isolated and cultured for in vitro lymphokine production.

Cell Culture Media

Roswell Park Memorial Institute medium 1640 (RPMI 1640)[1] was prepared according to manufacturer's instructions. Penicillin (100 units/ml) and streptomycin (100 gm/ml) were added and the pH was adjusted to 7.6. The medium was filter-sterilized through a 0.22 m millipore[2] filter and fetal bovine serum was added to a final con-centration of 5%.

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[1]Gibco Laboratories, Grand Island, New York.
[2]Millipore Corporation, Bedford, Massachusetts.

Medium-199 (M-199) was prepared according to manufacturer's instructions. Penicillin (100 units/ml), streptomycin (100 μgm/ml) and kanamycin (50 g/ml) were added and the pH was adjusted to 7.6. The medium was filter-sterilized with fetal bovine serum to give a final concentration of 20%.

Ficoll-Hypaque Density Gradient Solution

Ficoll-hypaque was used for isolation of lymphocytes. It was prepared by making a 9% (W/V) solution of Ficoll 400[1] in distilled water and a 34% (W/V) solution of sodium diatrizoate[2] (hypaque) in distilled water. A solution of ficoll-hypaque with a specific gravity of 1.078 was obtained by mixing 64 parts of the ficoll solution with 36 parts of the hypaque solution. After filter-sterilization (0.22 μm) the solution was stored at 4 C.

Preparation of Mitogen

Concanavalin A (Con-A)[2] was reconstituted with RPMI 1640 cell culture medium and was used at a rate of 0.15 μgm/well in the lymphocyte proliferation assay.

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[1]Pharmacia, Piscataway, New Jersey.
[2]Sigma Chemical Co., St. Louis, Missouri.

Peripheral Lymphocyte Collection and Processing

Blood samples were collected from all animals, except mice, into evacuated tubes containing EDTA as an anticoagulant (1.5 mg/ml blood). Alsever's solution (Alsever and Ainslie 1941) (1 ml Alsever's solution to 1 ml blood) was found to be a more satisfactory anticoagulant for collecting mouse blood. With the use of Alsever's solution during the collection of mouse blood, the amount of red blood cell contamination of lymphocyte suspensions was greatly reduced.

Lymphocytes were isolated from the uncoagulated blood sample by centrifugation through ficoll-hypaque. Blood samples were diluted 1:1 with Hank's balanced salt solution (HBSS), pH 7.6. Eight ml of the diluted blood were layered onto 4 ml of ficoll-hypaque in siliconized 17 x 100 mm plastic tube. Blood tubes were centrifuged at 400 x g for 30 min. Lymphocytes sedimented to the interface between the ficoll-hypaque and the plasma layer. The cell band was removed and placed in a siliconized 12 x 75 mm plastic tube with 5 ml of HBSS. This lymphocyte suspension was centrifuged at 200 x g for 10 min. The HBSS was aspirated and replaced with 5 ml of fresh solution. The cells were resuspended and pelleted once more by centrifugation at 200 x g for 10 min. The HBSS was aspirated and replaced with 1 ml of 2x M-199. A viable cell count was done using the trypan blue exclusion technique to differentiate between living and dead cells

Antigen stimulated lymphokine production was accomplished by mixing the 1 ml of lymphocyte suspension with 2 ml of a suspension of SAD rabies

virus, followed by incubation at 37 C for 72 h in a 5% CO_2 atmosphere. After incubation, the suspension was centrifuged at 400 x g for 10 min to remove cellular debris. The supernatant was removed and stored at - 20 C.

Preparation of Mouse Spleen Cells

Mice were killed by cervical dislocation, the abdominal area was drenched with 70% alcohol and the skin was removed. Mice from which serum was to be collected were anesthetized with ether and bled via the orbital sinus prior to being killed. Using sterile thumb forceps and scissors the peritoneal cavity was opened and the spleen was aseptically removed.

The spleen was placed in a sterile 60 mm tissue culture dish containing 5 ml of RPMI-1640 cell culture medium. Spleen cells were obtained by teasing the spleen apart with 2 20 ga. x 1 1/2 in. hypodermic needles. The cell suspension was gently aspirated and expelled through a 16 ga needle and then expelled through a 25μm mesh screen into a 17 x 100 mm plastic tube. The remaining spleen pulp was washed 2 additional times in 5 ml of RPMI-1640 medium.

The spleen cell suspension (about 7.5 ml/tube) was layered on 4 ml of ficoll-hypaque solution in a 17 x 100 mm plastic tube and centrifuged at 400 x g for 20 min. Lymphocytes were aspirated from the ficoll-hypaque medium interface and washed by centrifugation in RPMI-1640 medium at 200 x g for 10 min

Following the third wash the cells were resuspended in RPMI-1640

medium and the cell concentrations were determined using commercial

blood diluters[1] and a hemocytometer. Spleen cell suspensions consisted

of 95% or higher lymphocytes and the cell viability, as determined

with the trypan blue exclusion technique, was usually greater than 95%.

The cell concentration was adjusted to 5×10^6 viable cells/ml for the

proliferation assay and 10^8 viable cells/ml for TF production.

Gel Filtration

Partial purification of TF was accomplished using ascending flow

gel filtration through Sephadex G-10, G-25, or G-50[2] in a 9 mm x 600 mm

column. The Sephadex gels were hydrated in phosphate-buffered saline

pH 7.6. Three ml of lymphokine-rich supernatant were filtered through

the column at 4 C at a flow rate of 0.8 ml per min with phosphate

buffered saline (pH 7.6) as the eluant. Fractions were collected in

2 ml volumes using a LBK fraction collector and monitored at 280 mm.

The TF fractions were stored at -20 C.

Quantitative Assays of RNA and Protein

RNA determination

The RNA concentration of the TF preparations was measured using

the orcinol reaction described by Shatkin (1969). RNA standards were

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[1]Unipette, Becton, Dickinson & Co., Rutherford, New Jersey.
[2]Pharmacia Fine Chemicals, Piscataway, New Jersey.

prepared using Grade VI RNA derived from <u>Torula</u> yeast[1] and 5-methyl

resorcinol monohydrate[1] was used to prepare the orcinol reagent.

<u>Protein determination</u>

The protein concentration of the TF preparations was measured

using the Peterson (1977) modification of the Lowry technique.

Standard protein concentrations were prepared using bovine serum

albumin powder.[2]

<div align="center">Viral and Immuno Assays</div>

<u>Rabies specific fluorescent antibody test</u>

Impression smears were made from the cerebral cortex, hippocampus

and stem areas of the brain of some animals (excluding mice) which died

during rabies challenge experiments. A single horizontal cross section

was made through the brains of experimental mice which died during

<u>in vivo</u> studies of TF activity. Air-dried impression smears of the

brain sections were fixed in cold (-20 C) acetone for 30 min and

stored at -20 C prior to staining. The smears were stained with fluores-

cein labeled anti-rabies globulin[3] and examined with ultraviolet

microscopy for evidence of rabies antigen (Dean and Ableseth 1973).

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[1] Sigma Chemical Co., St. Louis, Missouri.

[2] Reheis Chemical Co., Chicago, Illinois.

[3] BBL Division of Becton, Dickinson and Company, Cockeysville, Maryland.

Antibody concentration assay

Rabies antibodies in serum samples obtained from immunized mice were titrated using the passive hemagglutination (PHA) procedure (Gough and Dierks 1971).

Virus concentration

The mouse inoculation test as described by Koprowski (1973a) was used for virus titration procedures in this study. Tenfold serial dilutions of the virus to be titrated were made in RPMI 1640 medium. Five mice were inoculated with 1 of 6 dilutions of a 20% (W/V) mouse brain suspension. Virus concentrations were calculated using the Spearman-Karber Method (Lorenz and Bogel 1973).

Indirect leucocyte migration inhibition (ILMI)

Indicator leucocytes   One volume of 6% dextran in 85% saline was added to 10 volumes of anticoagulated swine blood and the cell suspension was inverted and reverted 5 times. After gravity sedimentation for 30 min at 37 C the leucocyte-rich plasma layer was removed by aspiration, diluted with 3 volumes of a 0.83% solution of ammonium chloride to remove red blood cells, and held at room temperature for 5 min. The cell suspension was centrifuged at 200 x g for 10 min. The sedimented leucocytes were resuspended in 10 ml of Alsever's solution and centrifuged at 200 x g for 15 min. The wash with Alsever's solution was repeated once more. This procedure greatly reduced the amount of cell clumping observed as compared with that seen after washing cells with

other isotonic solutions. The cells were resuspended in M-199 and were counted. The viability was determined by using the trypan blue exclusion technique. The cell concentration was adjusted to 2×10^8 viable cells per ml.

Agar plates   Fresh 2% agar was prepared on the day the assay was to be run. The agar was hydrated in distilled water by heating in a 100 C water bath. Once rehydrated, the agar solution was placed in a 49 C water bath. Double strength M-199 was heated in the 49 C water bath. Equal volumes of the agar solution and the tissue culture medium were mixed thoroughly. Two and one-half ml of the M-199 solution was pipetted into each well (35 mm diameter) of 6 well tissue culture plates and allowed to solidify at room temperature. In order to increase the firmness of the agar, the tissue culture plates were refrigerated at 4 C until used. Just prior to being used each plate was removed from the cold and 3 holes (15 mm apart and 10 mm from one edge of the well) were punched in the agar with the use of a sterile 2.6 mm agar punch. The agar was removed from each hole with light suction through a sterile Pasteur pipette. Holes punched in cold agar were even edged consistent in size.

Indicator cell stimulation   Fifty μl of swine peripheral leucocyte suspension previously adjusted to 2×10^8 viable cells per ml and an equal volume of the supernatant from cell culture lymphocyte were placed in plastic tubes. This mixture was incubated at 37 C in a 5% CO_2 atmosphere for 90 min to allow for stimulation of indicator

cells. The positive control samples consisted of supernatant solution
from lymphocytes isolated from mice that had been inoculated with
CVS-27 rabies virus. The negative control sample was a mixture of
SAD rabies virus ($10^{7.5}$ MICLD50/ml) and an equal volume of M-199
medium.

   Incubation of migration inhibition plates   Following the 90 min
incubation of indicator leucocytes with the supernatant fluids, 10 ml
of cell suspensions were dispensed into the agar holes. Each 6
well tissue culture plate contained 5 test samples and 1 negative control
sample and each sample was tested in triplicate. Positive control
samples were dispersed throughout the group of test samples. All samples
were coded by number to ensure objectivity when measuring the distance
of migration. After the plates were incubated in a 5% CO_2 atmosphere
at 37 C for 24 hrs, each well was flooded with 2 ml of 7.5% glutaraldehyde
and held at room temperature for 45 min to fix the indicator cells.
The glutaraldehyde was rinsed from the wells with distilled water, the
agar was removed, the wells were rinsed gently with distilled water and
the plates were allowed to air dry. After they were thoroughly dried,
the wells were flooded with Giemsa stain for 30 min at room temperature.
This was followed by one rinse with tap water, and one rinse with
distilled water, and another drying cycle. During all drying cycles
the plates were placed on a slight incline to prevent pooling of water
over the cell layers which would result in a distinct water line after
staining.

Measurement of migration values   The distance of migration
was determined by measuring the distance from the perimeter of the agar
hole to the perimeter of the migrating cell line. Measurement was
accomplished with the use of a dissecting microscope equipped with an
ocular micrometer. The distance of cell migration for the 3 replicates
was compared statistically, using the Student's t-test, with the
migration observed in the 3 replicates of the negative control on the
same plate. A reduced mean migration between the test and negative
control wells, statistically significant at $P<0.05$ level, was considered
to be positive and to indicate the presence of leucocyte migration
inhibition factor in the test sample. Migration indices were calculated
by dividing the square of the mean distance of cell migration for the
test sample by the square of the mean distance of cell migration in
the negative control sample.

$$MI = \frac{(\text{mean distance of cell migration of test})^2}{(\text{mean distance of cell migration of negative control})^2}$$

Once t-tests were performed and migration indices were calculated the
numbered samples were decoded.

Lymphocyte proliferation assay

The lymphocyte proliferation assay used was a modification of the
procedure described by Valentine (1971). Spleen cell suspensions in
RPMI-1640 and adjusted to 5×10^6 viable cells/ml were prepared as
previously described. The cell cultures were set up in quadruplicate
in 96 well, 6.4 mm flat bottom tissue culture plates. In each well,

0.1 ml of the cell suspension (5 x 10^5 spleen cells) and 0.1 ml of the

previously determined optimum concentration of antigen or mitogen were

dispensed. Appropriate medium and spleen cell controls were included.

The cultures were incubated at 37 C in a 5% CO_2 humid atmosphere for

5 to 7 days, depending on the test variation being run. Five hours

after labeling with 1 µCi of ^{3}H-thymidine (specific activity 6.7 Ci/m

mol),[1] the cultures were freeze-thawed once and harvested with the use

of a multiple well aspirator[2] onto glass fiber filters.[2] The glass

fiber filter disks were placed in counting vials[3] containing 5 ml of

liquifleur solution and the amount of incorporated radiation was

determined with the use of a Beckman LS-230 liquid scintillation

ounter.[4]

Preparation of Dialyzable TF

Transfer factor was isolated using a modification of the procedure

described by Ascher et al. (1974). This consisted of TF prepared by

the conventional dialysis of lymphocyte extracts against distilled

water (TF_{DW}) for in vivo experiments and by dialysis against tissue

culture medium (TF_{DM}) for in vitro experiments. Mouse spleen cells

were collected as described earlier and were suspended in sterile

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[1]New England Nuclear, Boston, Massachusetts.
[2]Bellco, Vineland, New Jersey.
[3]Research Products International Corp., Mt. Prospect, Illinois.
[4]Beckman Instruments, Inc., Fullerton, California.

distilled water (2 ml per 10^8 viable spleen cells) containing 50 μgm/ml

of bovine pancreatic DNase.[1] Two ml aliquots were placed in sterile

12 x 75 mm plastic tubes and stored at -70 C. When needed for further

processing, the cells were subjected to 10 cycles of freezing in

dry ice-alcohol and thawing in a 37 C water bath. The lysate was

pipetted into a 0.64 cm diameter dialysis tubing[2] and the tubing was

placed in the dialysis fluid.

Dialysis of TF_{DW} To prepare the material for in vivo use,

2 ml of leucocyte lysate was dialyzed for 18 hours at 4 C against 200

volumes of sterile distilled water. The dialysate was shell frozen

in a dry ice-alcohol bath and lyophilized. The lyophilizate was

reconstituted in 3.3 ml of sterile distilled water (1 ml for each

3 x 10^7 spleen cells), filter-sterilized (0.22 μm millipore filter)

and frozen at -20 C until used.

Dialysis of TF_{DM} To prepare the material for in vitro use,

2 ml of leucocyte lysate was dialyzed for 18 hours at 4 C against

16.6 ml of RPMI 1640 medium (1 ml medium for every 6 x 10^6 spleen cells)

without serum. The dialysate was filter-sterilized (0.22 μm Millipore

filter) and stored at 4 C until used.

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[1]Sigma Chemical Co., St. Louis, Missouri.
[2]Arthur H. Thomas Co., Philadelphia, Pennsylvania.

Part 1. Preliminary Adoptive Immunization Experiments

Utilizing Antigen Stimulated TF Preparations

A profile of TF preparations used in all preliminary experiments is found in Table 4.

Transfer factor treatments in mice

A diagram of the treatment schedules of mice with TF is seen in Table 5.

Trial A: Homologous and heterologous treatment   Transfer factor preparations A (mouse origin) and C (hamster origin) were used to treat 2 groups of 30 mice each. Each group of mice was given TF 2 days pre-challenge and 3 and 6 days post-challenge with CVS-27 rabies virus. A third group of 30 mice was untreated and served as a control.

Tenfold dilutions of a 20% (W/V) suspension of rabid mouse brain ($10^{6.2}$ to $10^{1.2}$ MICLD50) were made in M-199 cell culture medium. The mouse brain suspensions were then titrated in each group of mice by IM inoculation of 0.05 ml into the left hind leg. Five mice in each group received each dilution.

Trial B: Homologous TF administered by different routes   Transfer factor preparation A of mouse origin was used to treat 2 groups of 30 mice each and a third group of 15 mice. The 30 mice in group 1 were inoculated per os with the use of a feeding needle. The second group of 30 mice was inoculated IP and the third group of 15 mice was

Table 4. Profile of TF preparations from cell culture supernatant

Preparation	Animal Species	Immun. virus	Days PI Cells Harvested[a]	Column Bed[b]	Protein Conc.
A	Mouse	MLV-HEP	12	G-25	47 μg/ml
B	Rat	CVS-27	7	G-50	113 μg/ml
C	Hamster	CVS-27	7	G-25	37 μg/ml
D	Guinea pig	MLV-HEP	10	G-25	44 μg/ml
E	Guinea pig	MLV-HEP	10	G-25	94 μg/ml
F	Hamster	CVS-27	7	G-50	60 μg/ml
G	Skunk	CVS-27	7	G-10 + G-25	77 μg/ml
H	Skunk	CVS-27	7	G-10 + G-25	115 μg/ml

[a]10^8 cells were cultured for 72 hr in the presence of rabies antigen (SAD).

[b]Sephadex gels.

Table 5. Transfer factor treatment and inoculation regimes used in adoptive transfer studies in mice

Experiment	TF Preparation	μg Protein per inoculation	Volume	Inoculation Route	Days Pre-challenge	Days Post-challenge	Group Number
A	A	2.35	0.05 ml	IM (RHL)[a]	2		
		1.41	0.03 ml	IM (RHL)		3, 6	1
A	C	1.85	0.05 ml	IM (RHL)	2		
		1.11	0.03 ml	IM (RHL)		3, 6	2
B	A	2.35	0.05 ml	Per os	3		1
	A	2.35	0.05 ml	IP	3		2
	A	2.35	0.05 ml	IM (RHL)	3		3
C	B	3.39	0.03 ml	Per os	3		1
			0.03 ml	Per os		2,4,6,8,10, 12,14	
C	B	3.39	0.03 ml	IP	3		2
			0.03 ml	IP		2,4,6,8,10, 12,14	
C	B	3.39	0.03 ml	IM (RHL)	3		3
			0.03 ml	IM (RHL)		2,4,6,8,10, 12,14	

	A	1.41	0.03 ml	IM (RHL)	0^b		1
D	A	1.41	0.03 ml	IC	0^b		2
	A	1.41	0.03 ml	IM (RHL)	0^b		
		1.41	0.03 ml	IM (RHL)		2	3
	F	1.8	0.03	IP	3,1	1,3,5,7,9, 11,13	1
E							
	F	1.8	0.03	IP	14,12,10, 8,6,4,2		3

[a] RHL - right hind leg.

[b] Inoculation of TF immediately preceded the inoculation of challenge virus.

inoculated IM into the RHL. A control group of 30 mice was untreated. All test mice were treated 3 days prior to being challenged with CVS-27 rabies virus.

Tenfold dilutions of a 20% (W/V) suspension of rabid hamster brain ($10^{6.2}$ through $10^{1.2}$ MICLD50) were made in M-199 culture medium. The challenge virus was titrated in each group of mice by IM inoculation (0.05 ml) of a dilution of the brain suspension. Mice in the third (IM) treatment group were inoculated with only the 3 highest dilutions of the virus.

Trial C: Intramuscular, intraperitoneal and oral treatment with TF given pre- and post-challenge with rabies virus   Transfer factor preparation B of rat origin was administered to 3 test groups of 18 mice each. A fourth group of 18 control mice was untreated. Groups number 1 (per os), number 2 (IP), and number 3 (IM) were treated with TF 3 days pre-challenge and on alternate days from day 2 through day 14 post-challenge with CVS-17 rabies virus.

Six tenfold dilutions of a 20% (W/V) suspension of rabid mouse brain ($10^{5.8}$ to $10^{0.8}$ MICLD50) were made in M-199 cell culture medium. The dilutions of mouse brain suspension were titrated in each of the 4 groups of mice by IM inoculation of 0.05 ml into the LHL. Each dilution of virus suspension was inoculated into 3 mice in each test or control group.

Trial D: Transfer factor treatment intracerebrally and at the site of virus challenge   Transfer factor preparation A of mouse origin was administered to 3 test groups of 18 mice each. Group

number 1 was inoculated IC, groups numbers 2 and 3 were inoculated IM

in the RHL. A fourth group of control mice was untreated.

Five twofold dilutions of a 20% (W/V) suspension of rabid mouse

brain ($10^{7.2}$ to $10^{5.7}$ MICLD50) were made in M-199 culture medium.

Immediately following the inoculation of TF the mouse brain suspensions

were titrated in each group of test and control mice by IM inoculation

of 0.05 ml into the RHL. Each dilution of challenge virus suspension

was inoculated into 3 mice in each group. Two days post-challenge

test group number 3 was given a second TF treatment at the site of

viral challenge.

Trial E: Enhancement of vaccine response with treatment with TF

Transfer factor preparation F of hamster origin was used to test if

the protection afforded mice with the administration of commercial

rabies vaccine (DEV) could be enhanced with the administration of TF

from rabid hamsters.

Four groups of 18 mice each were utilized. Group number 1 received

TF 3 and 1 days pre-challenge and every other day from day 1 through

day 13 post-challenge. Group number 2 was inoculated IP every second

day with 0.25 ml of a 1:10 dilution of a single dose vial of DEV from

day 13 through day 1 pre-challenge. Group number 3 was treated with

rabies vaccine in the same manner as group number 2, except that each

inoculation of vaccine was preceded by 24 hr with an IP inoculation

of TF. Group number 4 was untreated and served as a control. The

mice were inoculated IM in the RHL with 0.05 ml. Rabid mouse brain

was titrated in each of the 4 groups of mice. Five twofold dilutions

were made in M-199 culture medium from a 20% (W/V) suspension of brain

($10^{7.2}$ to $10^{5.7}$ MICLD50) and 3 mice in each group received each

dilution.

Transfer factor treatment in guinea pigs

A diagram of the TF treatments of guinea pigs is seen in Table 6.

Trial A: Treatment with homologous TF   Two adult guinea pigs

were inoculated IM into the left hind leg with TF preparation of guinea

pig origin 48 hr prior to IM challenge with a 20% (W/V) suspension

of CVS-27 rabies virus ($10^{5.7}$ MICLD50) of mouse origin.   Challenge virus

was inoculated IM into the RHL of the 2 TF treated guinea pigs and

2 untreated controls.

The brains and salivary glands of guinea pigs which died during

the course of the experiment were removed for examination using the

rabies FA test.   Impression smears were made from the hippocampus,

cerebral cortex, and stem areas of the brain and from a cross section

of the salivary gland.

Trial B: Treatment with homologous and heterologous TF factor

Four adult guinea pigs were inoculated IM into the LHL with TF prepara-

tion D of guinea pig origin and 4 adult guinea pigs were inoculated

IM into the LHL with TF preparation C of hamster origin.   Two untreated

guinea pigs served as controls.   A 1:2 dilution of challenge virus was

made in M-199 culture medium.   Three days after TF treatment 2 guinea pigs

from each treatment group and one control were inoculated IM into the RHL

with the $10^{6.9}$ MICLD50 challenge virus and the remaining animals were

inoculated similarly with $10^{6.6}$ MICLD50 of the virus.

Table 6. Transfer factor treatment of guinea pigs

Experiment	TF Preparation	Inoculation Dose		Inoculation Route	Inoculation Schedule		Group Number
		μg Protein per Inoc.	Volume		Days Pre-challenge	Days Post-challenge	
A	D	22	0.5 ml	IM LHL[a]	3	0	1
B	D	8.8	0.2 ml	IM LHL	3	0	1
	C	18.5	0.4 ml	IM LHL	3	0	2

[a]LHL = left hind leg.

Transfer factor treatments in raccoons and skunks

A diagram of the TF treatments of raccoons and skunks is seen in Table 7.

Trial A: Treatment of raccoons with heterologous TF   One raccoon was inoculated IM with TF preparation D of guinea pig origin, another with TF preparation E of guinea pig origin. A third raccoon was untreated and served as a control. Forty-eight hr after treatment, the 3 raccoons were inoculated with $10^{5.3}$ MICLD50 of CVS-27 rabies virus in a 20% (W/V) mouse brain suspension.

Impression smears were made of the hippocampus, cerebral cortex, and stem areas of the brain and from a cross-section of the salivary gland of animals dying during the course of the trial or at the termination of the experiment. These smears were examined with FA for confirmatory diagnosis of rabies.

Trial B: Treatment of striped skunks with homologous TF   Twelve adult striped skunks were arbitrarily assigned to 3 treatment groups of 4 skunks each. Group number 1 was inoculated with TF preparation G of skunk origin. Group number 2 was treated with preparation H of skunk origin and group number 3 was inoculated with 0.85% sodium chloride solution.

Forty-eight hr after treatment 2 skunks from each treatment group and 2 control animals were challenged with $10^{6.6}$ MICLD50 CVS-27 rabies virus as a 20% (W/V) mouse brain suspension inoculated IM into the LHL. The remaining skunks were challenged with a 1:2 dilution of the 20% (W/V) suspension inoculum ($10^{6.3}$ MICLD50).

Table 7. Transfer factor treatments of wildlife

| Expt. | Species | TF Preparation | Inoculation Dose | | Inoculation Route | Inoculation Schedule | | Group Number |
			g Protein per Inoc.	Volume		Days Pre-challenge	Days Post-challenge	
A	Raccoon	D	44	1 ml	IM	2	0	1
	Raccoon	E	94	1 ml	IM	2	0	2
B	Skunk	G	38.5	0.5 ml	IM (RHL)	2	0	1
	Skunk	H	57.5	0.5 ml	IM (RHL)	2	0	2

A pre-treatment and a pre-challenge blood sample were obtained by cardiac puncture. This blood sample was assayed by the indirect leucocyte migration inhibition technique for evidence of an enhanced cellular immune response. The blood sample was also examined by the PHA procedure for evidence of a humoral immune response to rabies virus.

The animals were observed for 51 days for evidence of rabies. The brains and salivary glands from the animals that died during the course of the study and from the animals that were killed at the termination of the trial were examined for rabies antigen by FA.

Part 2: In Vitro and In Vivo Studies Utilizing

Mouse Leukocyte Dialysates

In vitro stimulation of naive lymphocytes with TF

Five tenfold dilutions of TF_{DM} preparations from both immunized
and nonimmunized mice were made in RPMI-1640 medium. Spleen cells were
collected from unstimulated BALB/c mice in the manner described earlier.
The lymphocytes were isolated and adjusted to 5×10^6 cells/ml in
RPMI-1640 medium containing 5% fetal bovine sera (FBS) and antibiotics.

Ten aliquots of the cell suspension were sedimented by centrifugation
at 200 x g for 10 min and each cell pellet was resuspended in a dilution
of the TF_{DM} preparations. These cell suspensions were then tested using
the lymphocyte proliferation assay for evidence of enhanced proliferation
in the presence of rabies antigen. Appropriate medium, virus, and cell
controls were tested in conjunction with the assay. The cells were
cultured in the presence of TF_{DM} for 7 days as suggested by Ascher et al.
(1974) for in vitro TF lymphocyte stimulation.

In vitro assay of the in vivo transfer of immunity to rabies virus

Transfer factor for in vivo use was prepared as described earlier
from both rabies-immunized and nonimmunized mice. The RNA concentrations
of the preparations were adjusted to 10 µg/0.4 ml in sterile distilled
water.

Five mice in each of 3 groups were inoculated IP with 0.04 ml of TF from either the immunized mice or nonimmunized mice or with an equal volume of sterile distilled water.

Spleen cells were collected from each group of mice 2 days after treatment and tested for evidence of specific response to antigen using the lymphocyte proliferation assay.

Effect of varied doses of TF_{DW} on resistance to rabies viral infection

Transfer factor from immunized and nonimmunized mice was adjusted to concentrations of 0.5 µg, 1.0 µg, and 10 µg of RNA per 0.5 ml in sterile distilled water resulting in 2 types of TF_{DW} at 3 concentrations each. Twenty Swiss-Webster mice were inoculated IP with 0.4 ml of 1 of each of the 6 solutions. In addition, 20 mice were inoculated IP with 0.4 ml of sterile distilled water.

Three tenfold dilutions of a 20% (W/V) CVS-27 rabid mouse brain suspension were made in RPMI-1640 medium for titration in each treatment and control group of mice. Five mice in each group were inoculated IM into the RHL with 0.05 ml of each dilution 2 days post-treatment with TF_{DW}.

Impression smears of the brains of mice which died during the 21 day observation period were examined by FA to confirm a diagnosis of rabies.

RESULTS

Gel Filtration of Antigen-Stimulated Lymphokines

The chart recordings of the elution patterns of TF batches which were partially purified with the use of Sephadex columns are shown in Figures 1-6. The fractions collected and used for in vivo adoptive immunization trials are indicated. A considerable amount of variation in the patterns was noted for preparations from different lymphocyte cultures.

Protein and RNA Concentration of TF Preparations

The results of the assays for the concentration of protein and RNA, performed on the individual TF preparations, are listed in Table 8. These data were used to quantitate the dosage of TF used for adoptive stimulation both in vivo and in vitro.

Lymphocyte Proliferation Assay: Mitogen and

Antigen Stimulation of Lymphocytes

In order to determine that all spleen cell preparations were capable of being transformed in vitro, each culture of lymphocytes was stimulated with Con-A. The concentration of Con-A used to yield a maximum level of stimulation for the conditions of culture used during this study was determined (Table 9). High concentrations of Con-A were found to be

Figure 1. Chart recording of elution pattern of cell culture
supernatant from a Sephadex G-25 column of mouse lymphocytes
stimulated with MLV-HEP rabies virus and harvested 12 days
after inoculation

Figure 2. Chart recording of elution pattern of cell culture
supernatant from a Sephadex G-50 column of rat
lymphocytes stimulated with CVS-27 rabies virus and
harvested 7 days after inoculation

Figure 1

Preparation

Figure 2

Preparation
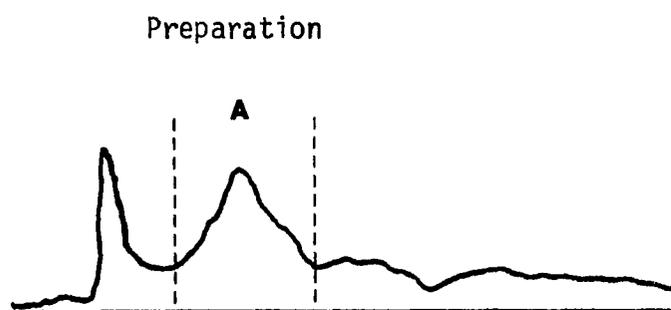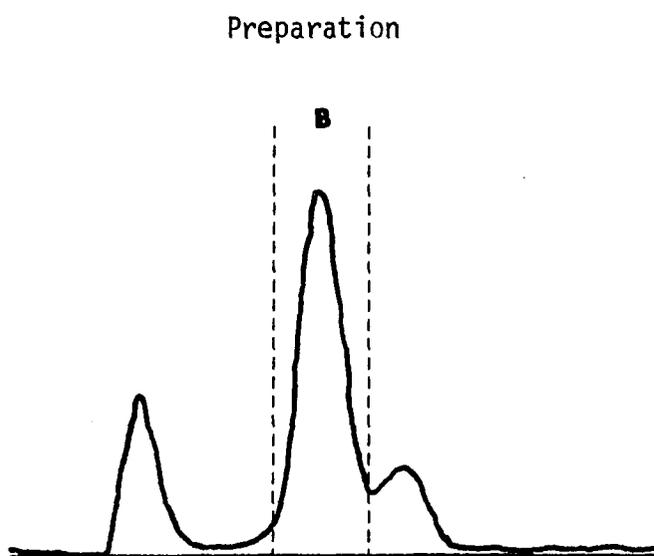
Figure 3. Chart recording of elution pattern of cell culture
supernatant from a Sephadex G-25 column of hamster
lymphocytes stimulated with CVS-27 rabies virus and
harvested 7 days after inoculation

Figure 4. Chart recording of elution pattern of cell culture
supernatant from a Sephadex G-25 column of guinea pig
lymphocytes stimulated with MLV-HEP virus and
harvested 10 days after inoculation

Figure 3

Preparation

Figure 4

Preparations
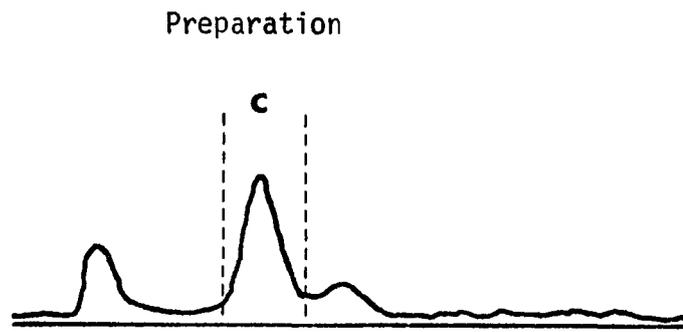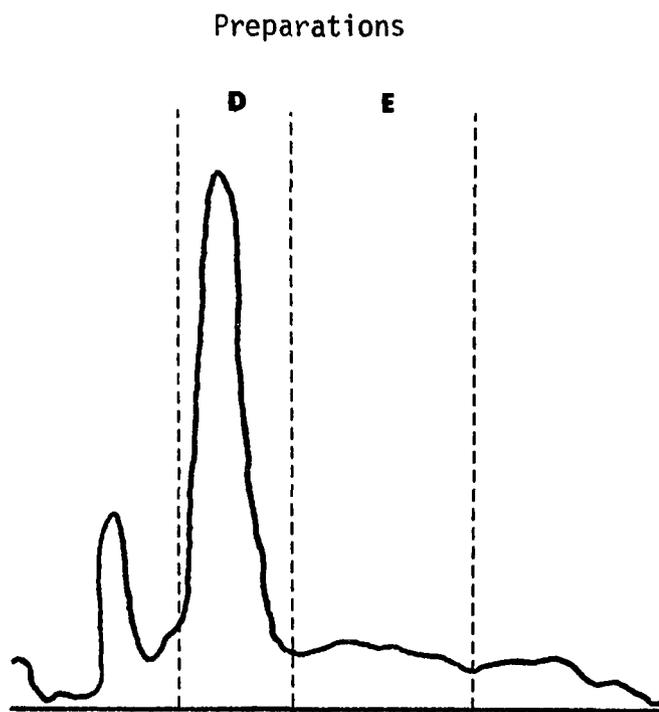
Figure 5. Chart recording of elution pattern of cell culture

supernatant from a Sephadex G-50 column of hamster

lymphocytes stimulated with CVS-27 rabies virus and

harvested 7 days after inoculation

Figure 6. Chart recording of elution pattern of cell culture

supernatant from Sephadex G-10 and Sephadex G-25

columns in series. Peripheral lymphocytes from a

striped skunk were stimulated with CVS-27 rabies virus

prior to harvest at 7 days

Figure 5

Preparation
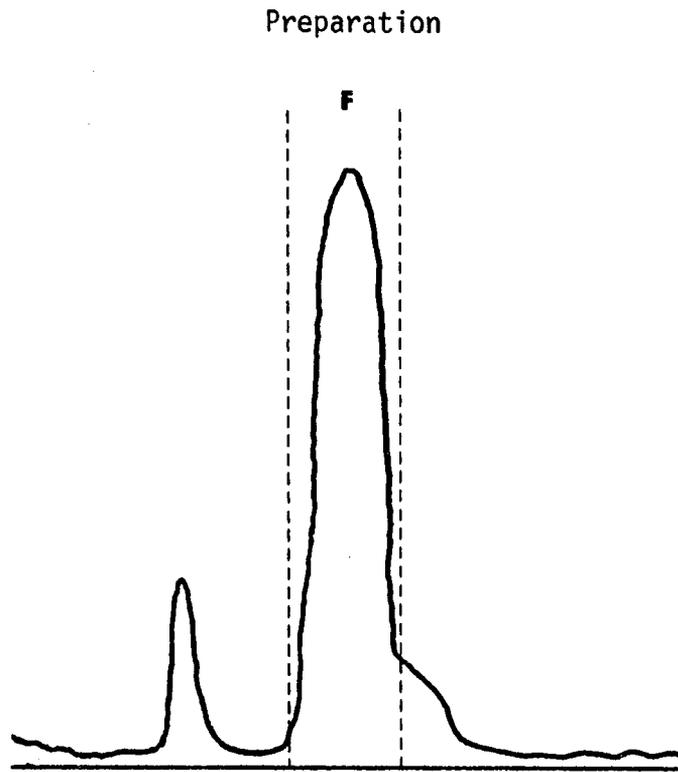
Figure 6
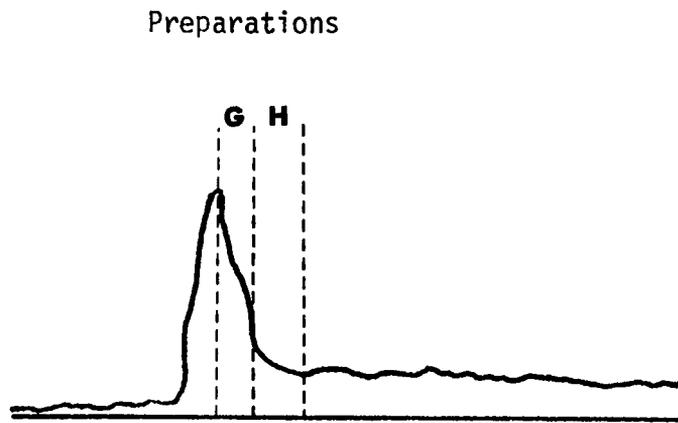
Preparations

Table 8. Protein and RNA concentration of TF preparations

Purification Procedure	TF Preparation	Source of Lymphocytes	Protein[a] Concentration μg/ml	RNA [b] Concentration ug/ml
Gel Filtration	A	Mouse Peripheral	47	ND[c]
	B	Rat Peripheral	113	ND
	C	Hamster Peripheral	37	ND
	D	Guinea Pig Peripheral	44	ND
	E	Guinea Pig Peripheral	94	ND
	F	Hamster Peripheral	60	ND
	G	Skunk Peripheral	77	ND
	H	Skunk Peripheral	115	ND
Dialysis	I	Mouse Spleen	160	54
	J	Mouse Spleen	80	25
	K	Mouse Spleen	175	41
	L	Mouse Spleen	100	23

[a]Lowry Reaction.

[b]Orcinol Reaction.

[c]ND = Not Done.

Table 9. ^{3}H-thymidine incorporation of mouse spleen cell lymphocytes and stimulation index after exposure to varying concentrations of Con-A

Con-A Concentration ug/ml	Average CMP[b]	SI[a]
150	5.0	0.01
15	719.5	1.03
1.5	3337.6	4.80
.15	5949.5	8.55
.015	944.8	1.36
.0015	519.5	0.85
.00015	503.3	0.72
0	695.5	

[a] $\dfrac{\text{Average counts per minute of the stimulated culture.}}{\text{Average counts per minute of the control culture.}}$ = Stimulation Index

[b] Total counts per minute minus counts per minute of background media culture.

toxic to the cultures. The optimum concentration of Con-A added to each cell culture was 0.15 g/ml.

Spleen cells from mice immunized by foot pad inoculation of DEV rabies vaccine were cultured in the presence of tenfold dilutions of purified SAD virus to determine the optimum concentration of antigen necessary to be able to detect a specific cellular response to rabies virus. Control cultures consisted of: 1) Spleen cells from nonimmunized mice, used to detect nonspecific stimulation; 2) Con-A, used to promote nonspecific stimulation; 3) Spleen cells cultured in the presence of medium without antigen, used to determine the amount of ^{3}H-thymidine to be incorporated in unstimulated cells and 4) Medium cultures, made to quantitate the amount of background radiation that was attributable to the medium.

As illustrated in Table 10, stimulation of spleen cells from immunized mice was directly related to the concentration of antigen used. A maximum stimulation was detected in microtiter cell cultures inoculated with a $1:10^3$ dilution of the purified rabies virus preparation.

Adoptive Transfer Experimentation in vivo: Preliminary Studies

Mice

Trial A: Homologous and heterlogous treatment   Titers of rabies virus in the 3 groups of mice were $10^{3.6}$ IMLD50 for the homologous TF treatment group, $10^{2.0}$ IMLD50 for the heterlogous TF treatment group,

Table 10. ^{3}H-thymidine incorporation of mouse spleen cell lymphocytes and stimulation index after exposure to varying concentrations of rabies antigen

Rabies Antigen Dilution	Average CPM [a]		SI [b]	
	Normal Cells	Immune Cells	Normal Cells	Immune Cells
1:10	616.8	432.6	1.37	1.10
1:10^2	562.2	1707.2	1.25	4.34
1:10^3	697.1	2673.0	1.55	6.80
1:10^4	562.4	2205.4	1.25	5.61
1:10^5	558.4	1049.6	1.24	2.67
1:10^6	569.5	750.8	1.26	1.91
0	450.4	393.3		

[a]Total counts per minute minus counts per minute of background media culture.

[b]$$\frac{\text{Average counts per minute of the stimulated culture.}}{\text{Average counts per minute of the control culture.}} = \text{Stimulation Index}$$

and $10^{3.2}$ IMLD50 for the control group. All mice dying after exhibiting clinical signs of rabies died between 6 and 9 days post-challenge.

Trial B: Homologous TF administered by different routes

Titers of rabies virus in 3 of the groups of mice were $10^{1.17}$ MICLD50 for both those receiving TF either per os or IP and for the control animals. A virus titer could not be determined for those which received TF by the IM route because the 50% endpoint was found to be below the lowest dilution of virus ($10^{3.2}$ MICLD50) administered to the mice.

Trial C: IM, IP and oral treatment with TF given pre- and post-challenge with rabies virus   Control mice inoculated with $10^{4.5}$ MICLD50 of CVS-27 rabies virus died of rabies. This was the highest concentration of viral challenge. None of the mice in the 3 TF treatment groups died during the course of the trial.

Trial D: TF treatment IC and at the site of virus challenge

Mice died in all treatment groups during this trial. As seen in Table 11, deaths in the TF treated groups of mice occurred earlier than they did in the control groups. The percent mortality for each treatment and control group of mice is indicated in Table 12. Prior to day 9 post-challenge 17% of the IC treated mice, 22% of the IM x 1 group, and 28% of those treated with 2 doses of TF had died of rabies as compared with 0% deaths in the untreated control group. Mortality rates for mice receiving TF were essentially equal to or greater than the mortality rate of control mice.

Table 11. Periodicity of mouse mortality following treatment with TF and subsequent challenge with rabies virus

TF Treatment	Challenge[a] Virus Dose MICLD50	6[b]	7	8	9	10	11	12	13
IC	$10^{5.9}$	-[c]	-	D[d]	DD				
	$10^{5.6}$	D	-	D	D				
	$10^{5.3}$	-	-	-	-	-	-	-	-
	$10^{5.0}$	-	-	-	D	-	-	-	-
	$10^{4.7}$	-	-	-	D	-	-	-	-
	$10^{4.4}$	-	-	-	-	-	-	-	-
IM x 1[e]	$10^{5.9}$	-	-	DD	-	-	-	-	-
	$10^{5.6}$	-	-	D	-	D	D	-	-
	$10^{5.3}$	-	-	D	D	-	-	-	-
	$10^{5.0}$	-	-	-	D	-	-	-	-
	$10^{4.7}$	-	-	-	D	D	-	-	-
	$10^{4.4}$	-	-	-	D	-	-	-	-
IM x 2[f]	$10^{5.9}$	-	-	D	D	-	D		
	$10^{5.6}$	-	-	-	DDD				
	$10^{5.3}$	-	-	DDD					
	$10^{5.0}$	-	-	-	-	-	-	-	D
	$10^{4.7}$	-	-	-	-	D	-	-	-
	$10^{4.4}$	D	-	-	D	-	-	D	
Control	$10^{5.9}$	-	-	-	D	D	-	-	-
	$10^{5.6}$	-	-	-	-	-	D	D	-
	$10^{5.3}$	-	-	-	D	D	-	-	-
	$10^{5.0}$	-	-	-	D	-	-	D	-
	$10^{4.7}$	-	-	-	D	-	-	-	-
	$10^{4.4}$	-	-	-	-	-	-	-	-

[a] 3 mice in each treatment and control group were inoculated with each challenge virus dilution.

[b] No deaths occurred before 6 days.

[c] - = no mouse deaths.

[d] D = mouse died from rabies infection.

[e] IM x 1 = Treated with TF at the site of virus inoculation immediately preceding challenge.

[f] IM x 2 = Treated with TF at the site of virus inoculation immediately preceding challenge and 2 days post-challenge.

Table 12.   Mortality rate of mice treated with TF IC and at the
site or rabies virus challenge

TF Treatment[a]	Days Post-Challenge								Cumulative Total
	6[b]	7	8	9	10	11	12	13	
IC	1/18[c]	0/18	2/18	5/18	0/18	0/18	0/18	0/18	8/18
(%)	(6)	(0)	(11)	(28)	(0)	(0)	(0)	(0)	(44)
IMx1	0/18	0/18	4/18	4/18	2/18	1/18	0/18	0/18	11/18
(%)	(0)	(0)	(22)	(22)	(11)	(6)	(0)	(0)	(61)
IMx2	0/18	0/18	4/18	5/18	1/18	1/18	1/18	1/18	14/18
(%)	(6)	(0)	(22)	(28)	(6)	(6)	(6)	(6)	(78)
Control	0/18	0/18	0/18	4/18	2/18	1/18	2/18	0/18	9/18
(%)	(0)	(0)	(0)	(22)	(11)	(6)	(11)	(0)	(50)

[a]M = 18 for each treatment group.

[b]No mice died prior to 6 days post-challenge.

[c]Number of mice dead.

———————————————————————————————————

Number of mice treated and challenged.

Trial E: Enhancement of vaccine response following treatment with TF

Table 13 is an outline of the mortality observed in the various treatment groups of mice and the control group following challenge with rabies virus. All mice treated with rabies vaccine plus TF and those treated only with rabies vaccine survived challenge. Mortality due to rabies was observed in both the control group and the group receiving TF only.

All mice receiving TF died of rabies following challenge with $10^{5.9}$ and $10^{5.6}$ MICLD50 of the virus. One of the 3 mice treated with TF only and challenged with $10^{5.3}$, $10^{5.0}$, $10^{4.7}$ and $10^{4.4}$ MICLD50 of rabies virus died of rabies. Two of 3 untreated control mice died of rabies subsequent to challenge with $10^{5.9}$, $10^{5.6}$, $10^{5.3}$ and $10^{5.0}$ MICLD50 rabies virus; 1 of 3 control mice died after challenge with $10^{4.7}$ MICLD50 and none died following challenge with $10^{4.4}$ MICLD50. As observed in the preceding experiment, mice treated with TF had an earlier onset of death than did untreated control mice.

Antibody titers of pooled blood samples collected at the termination of this trial are listed in Table 14. Rabies antibody was not detected in sera from mice that received TF alone or in sera collected from untreated control mice. Rabies specific antibody was detected in the 2 groups (those administered vaccine plus TF and those given vaccine alone) of mice which had been injected with rabies vaccine. No significant difference (P .05) in antibody titers was seen in sera from the 2 groups of mice.

Table 13. Periodicity of mouse mortality following treatment with TF and/or rabies vaccine and subsequently challenged with rabies virus

Treatment	Challenge[a] Virus Dose MICLD50	Days Post-Challenge					
		7[b]	8	9	10	11	12
TF only	$10^{5.9}$	D[c]	D	D			
	$10^{5.6}$	-[d]	-	DDD			
	$10^{5.3}$	-	-	-	D	-	-
	$10^{5.0}$	-	-	D	-	-	-
	$10^{4.7}$	-	-	D	-	-	-
	$10^{4.4}$	-	-	-	D	-	-
Vaccine only	$10^{5.9}$	-	-	-	-	-	-
	$10^{5.6}$	-	-	-	-	-	-
	$10^{5.3}$	-	-	-	-	-	-
	$10^{5.0}$	-	-	-	-	-	-
	$10^{4.7}$	-	-	-	-	-	-
	$10^{4.4}$	-	-	-	-	-	-
TF + Vaccine	$10^{5.9}$	-	-	-	-	-	-
	$10^{5.6}$	-	-	-	-	-	-
	$10^{5.3}$	-	-	-	-	-	-
	$10^{5.0}$	-	-	-	-	-	-
	$10^{4.7}$	-	-	-	-	-	-
	$10^{4.4}$	-	-	-	-	-	-
Control	$10^{5.9}$	-	-	D	D	-	-
	$10^{5.6}$	-	-	-	-	D	D
	$10^{5.3}$	-	-	D	D	-	-
	$10^{5.0}$	-	-	D	-	-	D
	$10^{4.7}$	-	-	D	-	-	-
	$10^{4.4}$	-	-	-	-	-	-

[a] 3 mice in each treatment and control group were inoculated with each challenge virus dilution.

[b] No mice died prior to day 7 post-challenge.

[c] D = mouse died from rabies infection

[d] - = no mouse deaths.

Guinea pigs

Trial A: Treatment with homologous TF   Three of 4 guinea

pigs died by 8 days post-challenge. These 3 included both control

animals and 1 guinea pig treated with TF. As seen in Table 15,

rabies antigen was identified by rabies FA in tissues from all animals

dying during the course of this trial. The 1 remaining TF-treated

guinea pig remained healthy for more than 1 year post-challenge.

Trial B: Treatment with homologous and heterlogous TF   All

guinea pigs used in this trial died of rabies as determined by clinical

signs of the disease and rabies FA examination of impression smears

made from brain tissues.. The guinea pigs died within 5 to 7 days after

challenge with rabies virus.

Raccoons

Twenty-seven days post-challenge the control raccoon died of

rabies as diagnosed from clinical observations and confirmed by rabies

FA staining of tissue impression smears. Smears of the hippocampus,

brain stem and cerebral cortex were highly positive for the antigen

but the salivary gland was found to be negative. No clinical signs

of rabies were observed in the 2 raccoons receiving TF and both survived

until termination of the trial at 48 days post-challenge. Impression

smears of brain and salivary gland tissues were negative for rabies

antigen as determined by specific FA staining.

Table 14. Antibody titer[a] of pooled[b] blood samples[*]

Treatment	Challenge virus dose (MICLD50)					
	$10^{5.9}$	$10^{5.6}$	$10^{5.3}$	$10^{5.0}$	$10^{4.7}$	$10^{4.4}$
TF	ND[c]	ND	Neg[d]	Neg	Neg	Neg
Vaccine	1:8	1:16	1:2	1:4	1:2	1:2
TF + Vaccine	1:4[b]	1:16	1:8	1:2	1:8	1:8
Control	Neg	Neg	Neg	Neg	Neg	Neg

[a] Antibody titer determined using the rabies PHA test.

[b] Pooled sample of blood from mice in each group of 3 challenged with rabies virus which survived until the termination of the experiment.

[c] ND = not done.

[d] Neg = no detectable rabies antibody.

[*] No significant difference in geometric mean titers between mice treated with vaccine alone and mice treated with TF plus vaccine (P .05).

Table 15. Results of rabies FA examination of guinea pig brain and salivary gland impression smears

TF Treatment	Died Days PC[e]	Fluorescence			
		Hipp.[a]	C.C.[b]	B.S.[c]	S.G.[d]
Preparation D	8	1+	1+	Neg	Neg
Preparation D	-	-	-	-	-
Control	8	1+	Neg	Neg	Neg
Control	8	3+	3+	4+	1+

[a] Hipp. = hippocampus.

[b] C.C. = cerebral cortex.

[c] B. S. = brain stem.

[d] S. G. = salivary gland.

[e] PC = post-challenge inoculation.

Striped skunks

The results of TF treatment of skunks are listed in Table 16.
The two skunks (671 and 667) which had detectable rabies antibody and 4
animals (182, 675, 669 and 672) which had detectable cellular responses
in blood samples taken prior to treatment with TF were excluded from
further consideration in this study. One of 3 remaining animals treated
with TF preparation G and 1 of 1 remaining skunks treated with TF
preparation H died of rabies as diagnosed by clinical and rabies FA
testing. Both of these skunks had been challenged with $10^{6.6}$ MICLD50
rabies virus. None of the 2 remaining control skunks died of rabies.

Two of 3 (664 and 665) skunks which received TF preparation G
reverted from a negative to a positive cellular immune response
2 days after treatment. These 2 animals were challenged with $10^{6.3}$
MICLD50 rabies virus as was the control skunk challenged with this
dose of virus.

Adoptive Transfer Experimentation in vitro

Effects of TF and antigen on unsensitized lymphocytes

Lymphocyte proliferation assays were performed on normal mouse
spleen cells cultured in the presence at TF_{DM} and antigen to determine
if TF derived from immunized mice could specifically stimulate lymphocytes
from nonimmunized mice in vitro. As seen in Table 17, no specific or
nonspecific stimulation of the lymphocytes was noted for nonstimulated
lymphocytes cultured with antigen from either TF_{DM} nonimmunized mice

Table 16. Comparison of pre- and post-treatment humoral and cellular immune responses in skunks

Treatment	TF Preparation G				TF Preparation H				Control			
Challenge	$10^{6.6}$ MICLD50		$10^{6.3}$ MICLD50		$10^{6.6}$ MICLD50		$10^{6.3}$ MICLD50		$10^{6.6}$ MICLD50		$10^{6.3}$ MICLD50	
Animal Number	182^a	663	664	665	666	667^a	675^a	669^a	670	671^a	672^a	673
PHA[b]	Neg/ ND^c	1:2/ 1:2	Neg/ 1:2	Neg/ Neg	Neg/ Neg	1:4/ ND	1:2/ ND	Neg/ ND	Neg/ 1:2	1:4/ ND	Neg/ ND	Neg/ 1:2
MI[d]	0.65+/* ND	1:31/ 0.99	1:11/ 0.78+	0.94/ 0.67+	1.06/ 1.05	0.95/ ND	0.71+/ ND	0.56+/ ND	0.83/ 1.02	1.05/ ND	0.55+/ ND	0.89/ 1.32
FA[e]	ND	+	Neg	Neg	+	ND	ND	ND	Neg	ND	ND	Neg

[a] Eliminated from the study because of positive initial humoral or cellular immune response.

[b] PHA = passive hemagglutination; a titer of 1:4 is considered positive. $\left(\dfrac{\text{day of treatment assay}}{\text{day of challenge assay}}\right)$

[c] ND = not done.

[d] MI = migration index. $\left(\dfrac{\text{day of treatment assay}}{\text{day of challenge assay}}\right)$

[e] FA = Rabies specific fluorescent antibody assay of brain impression smears.

*Significantly positive (P .05).

Table 17. Stimulation indices of normal mouse lymphocytes cultured in the presence of TF_{DM} and rabies virus

TF_{DM} Dilutions	Virus Dilutions									Con-A[a]
	1:10	$1:10^2$	$1:10^3$	$1:10^4$	$1:10^5$	$1:10^6$	$1:10^7$	$1:10^8$	$1:10^9$	
TF_{DM} from immunized mice										
1:10	1.4	1.7	1.6	1.7	1.8	1.6	1.4	1.4	1.0	8.6
$1:10^2$	1.0	1.2	1.0	1.3	1.7	1.4	1.3	1.0	1.3	7.1
$1:10^3$	1.3	2.1	2.2	2.0	1.4	1.5	1.2	1.1	1.3	10.2
$1:10^4$	1.0	1.2	1.2	1.3	1.1	1.4	1.7	1.4	1.4	7.1
$1:10^5$	1.5	2.3	1.9	1.8	1.6	1.5	1.0	1.2	1.3	7.8
TF_{DM} from nonimmunized mice										
1:10	2.0	2.3	1.7	2.0	1.8	1.5	2.4	1.6	1.1	10.3
$1:10^2$	1.5	2.0	1.3	1.4	1.3	1.5	1.6	1.6	1.4	5.5
$1:10^3$	1.6	1.8	1.6	1.6	1.7	1.5	1.5	1.3	0.9	8.7
$1:10^4$	1.0	1.5	1.4	1.5	1.2	1.0	1.1	1.0	1.2	7.3
$1:10^5$	1.4	1.7	1.3	1.3	1.2	1.3	1.1	1.2	1.0	9.9

[a]Con-A concentration = 0.15 µg per culture well.

or TF_{DM} from immunized mice.

Adoptive Transfer Experimentation in vivo

Effect of TF on lymphocyte proliferation response

In order to determine if lysate TF from mice immunized with

rabies virus could enhance the cellular immune response to rabies virus

in vivo, inbred mice were treated with TF by IP inoculation with either

TF or with an equal volume of saline. Transfer factor derived from

immunized mice TF_{DW+} and nonimmunized mice (TF_{DW-}) was used in order

to identify any nonspecific lymphocyte stimulation or inhibition.

Table 18 is an outline of the results of the proliferation assays

performed on the 3 treatment groups of mice. Lymphocytes from mice

treated with either TF_{DW+} or TF_{DW-} had significantly higher average

incorporation of ^{3}H-thymidine than did lymphocytes from mice treated

with an equal volume of saline. In addition, the SI of cells from

TF_{DW+} treated mice cultured in the presence of antigen dilutions

($1:10^2$ through $1:10^5$) were significantly ($P<.001$) higher than those

observed from the similarly treated culture of cells from TF_{DW-} and

saline treated mice. Significance was determined by the Student's

t-test for unpaired means utilizing the count per minute readings and

the stimulation indices of the individual replicate cell cultures.

Table 18. ^{3}H-thymidine incorporation of mouse lymphocytes after in vivo treatment with TF or saline

Antigen	Average CPM[a]			SI's[b]		
	TF$_{DW+}$[c]	TF$_{DW-}$[d]	Saline	TF$^+_{DW}$	TF$^-_{DW}$	Saline
0	12423.8*	12267.0*	682.4			
1:10	8723.9	2214.2	330.5	0.7	0.2	0.5
1:10^2	29335.4	6314.4	934.0	2.4**	0.5	1.4
1:10^3	34491.6	9768.9	928.5	2.8**	0.8	1.4
1:10^4	33766.1	16128.5	996.5	2.7**	1.3	1.5
1:10^5	33165.5	20578.0	862.0	2.7**	1.7	1.3
1:10^6	18816.9	16127.9	803.4	1.5	1.3	1.2
1:10^7	12618.6	14430.4	669.6	1.0	1.2	1.0
1:10^8	13426.7	15732.6	787.5	1.1	1.3	1.2
1:10^9	12271.8	8931.2	692.6	1.0	0.7	1.0

[a] CPM = Counts per minute minus media background.

[b] $\dfrac{\text{Average counts per minute of the stimulated cell cultures}}{\text{Average counts per minute of the unstimulated cell cultures}}$ = Stimulation Index

[c] Transfer factor derived from immunized mice.

[d] Transfer factor derived from nonimmunized mice.

*Significantly higher (P .001) average CPM than CPM for lymphocytes from saline treated mice.

**Significantly higher (P .001) SI than determined for the stimulation of either TF-$_{DW}$ treated or saline treated mice.

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Effect of TF on resistance to infection with rabies virus

Swiss-Webster mice were treated with TF_{DW+} or TF_{DW-} by IP inoculation of 10, 1 or 0.5 µg of RNA in 0.4 ml of saline. A control group of mice was inoculated with 0.4 ml of saline only. After 2 days the mice were challenged with CVS-27 rabies virus inoculated IM into the RHL. As can be seen in Figure 7, there was no protection afforded to mice as a result of having been treated with either of the sources of TF. All mice which died during the course of this experiment died of rabies, as determined with the rabies FA procedure.

Figure 7. Mortality of TF_{DW} treated mice after challenge with CVS-27 rabies virus (N = 5)

▨ Treated with TF (RNA) from immunized mice

☐ Treated with TF (RNA) from nonimmunized mice.

◪ Treated with saline
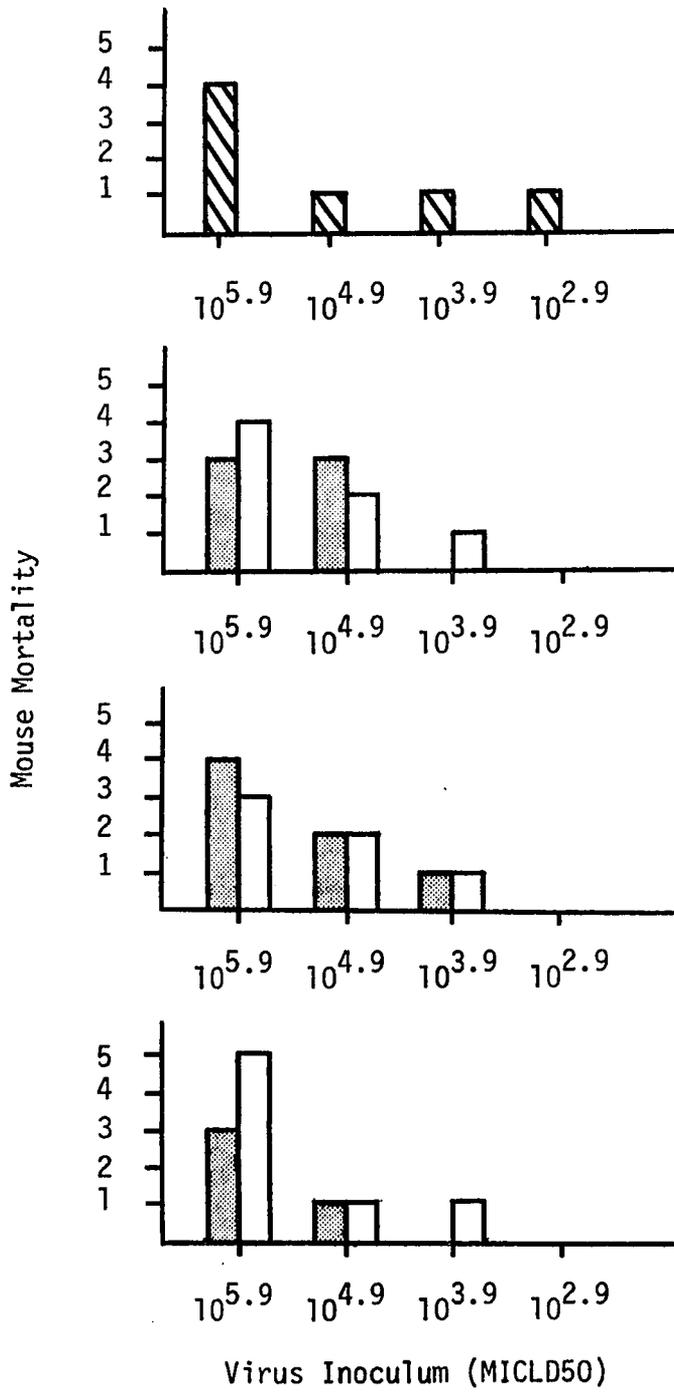
DISCUSSION

The transfer of lymphocytes or extracts of lymphocytes from sensitized donors to naive recipients has been used in man and other mammals to transfer immunity adoptively against a wide variety of pathogens. In early studies of the association of cellular immunity with rabies viral infections, Turner (1973) was unable to demonstrate any protection from rabies in mice which had been injected with splenocytes from syngeneic, immunized mice. More recently, however, Prabhackar, cited by Shope et al. (1979), was successful in transferring the resistance. Lagrange et al. (1978), using the foot pad test, showed that an in vivo CMI occurred in mice after adoptive immunization with sensitized splenocytes. Wiktor et al. (1974) and Hill (1974) observed in vitro lymphocyte blastogenesis in rabies-stimulated cell cultures from immunized rabbits and mice, respectively. Wiktor et al. (1974) and Wiktor et al. (1977a) also successfully demonstrated cell-mediated cytotoxicity using immune lymphocytes from mice and rabbits in a chromium release assay.

All published reports of adoptive transfer of immunity to rabies virus have involved the use of whole cells inoculated into syngeneic mice. In addition, in vitro demonstrations of cellular immunity have involved the stimulation of viable lymphocytes obtained from immunized animals. Although the occurrence of protection from rabies viral infection after the adoptive transfer of splenocytes is an interesting

phenomenon, the practical application of this technique is extremely limited due to the immunorejection reactions in other than syngeneic systems.

Theoretically, with the use of the dialyzable leucocyte extract, TF animals could be adoptively immunized against rabies and thus bypass probblems associated with immunorejection. Also, because the use of TF is not limited by allotypic barriers, it could be stimulated in domestic animals, for general use. A third major advantage of the use of TF is that adoptive transfer of immunity would not require immunization with the virus.

The purpose of this study has been to test the efficacy of TF preparations for immunization of laboratory and wildlife animal species. The activity of TF was also tested in vitro using the lymphocyte proliferation assay. The TF preparations were partially purified either by dialysis or with the use of gel filtration through Sephadex G-25 or G-50. Two columns, 1 containing Sephadex G-10 and the second G-25, were run in series to purify TF preparations G and H (Table 4). Because Lawrence et al. (1963) found that the fraction collected at peak 2, after filtration through Sephadex G-25, was able to transfer coccidioidin sensitively whereas the peak 1 components failed, the second component eluted in gel filtration also was selected for use in these experiments.

Dialysis is widely used today for purification of TF. The techniques described by Ascher et al. (1974) and Salaman (1978) were used as a model for this means of purification of some TF preparations.

In preliminary studies, a low level of resistance to challenge with rabies virus was seen in mice treated with heterologous TF of hamster origin in Trial A. The protection observed after pre- and post-exposure treatment with a total of 4.07 µg of preparation C was only 15.8 IMLD50 above the resistance seen in untreated control mice and can not be considered significant. Mice treated with homologous TF had a slightly higher mortality rate than was observed in the untreated control group but this increased susceptibility also is insignificant and possibly due to biological variation among the individual animals.

It should be noted that mice receiving homologous TF received a greater protein concentration than did mice which were treated with the heterologous preparation. Using the skin test response to tuberculin, Welch et al. (1976) observed a reduction in the number of responding animals after increased dosages of TF were administered, but Vandenbark, et al. (1976), using the same test system, were unable to induce any reduced skin test response after the administration of as much as 10 times the minimum level of TF needed to transfer reactivity to tuberculin. It was decided to repeat Trial A, using homologous TF preparation A at a lower dosage level. The added dimension of various routes of TF administration also was incorporated into Trial B. Lawrence (1974) reported that IV, IP, and IM routes of administration were equally effective when used to immunize guinea pigs adoptively against tuberculin and Jeter et al. (1977) reported the successful transfer of sensitivity to tuberculin in human recipients after TF administration per os.

The infectivity of the challenge virus, inoculated IM, was lower than was expected and deaths of mice in both groups were insufficient for a valid test of the relative resistance of treated and control mice. For this reason, the experiment was repeated with the use of a different batch of TF and a different batch of rabies virus inoculum.

Trial C involved mice that received TF both pre- and post-challenge with rabies virus. As in Trial B, the infectivity of the challenge virus inoculated IM was lower than was expected. However, control mice challenged with $10^{4.5}$ LD50 of CVS-27 rabies virus died of rabies and all treated mice were resistant to rabies at this level of challenge. None of the treated or control mice died of rabies after challenge with higher dilutions of the virus inoculum.

If the use of TF was found to be an efficacious immunizing agent against rabies in wild animal species, oral inoculation of wild populations with TF incorporated in food baits may be an efficient method of adoptive immunization. Because 9 of 9 treated mice survived while 3 of 3 untreated mice died when challenged IM with $10^{4.5}$ LD50 of the virus inoculum, it appears that TF treatments may have resulted in some increased resistance to infection with rabies virus.

It is believed to be desirable to develop a means of immunizing wildlife against rabies because the disease is endemic in populations of sylvatic animals in the United States, with occasional spill over into populations of domestic animals and humans. Investigations into the feasibility of using TF adoptively to immunize wildlife involved the use of raccoons and striped skunks, both of which pose significant

public health problems due to rabies in this country.

In the first such trial, 2 raccoons were treated with different TF obtained from the same guinea pig lymphoid cell culture supernate. A third raccoon was untreated and served as a control. At 48 hrs post-treatment, all 3 raccoons were inoculated IM with $10^{5.3}$ MICLD50 of rabies street virus of skunk origin. The control raccoon died of rabies 27 days post-challenge and rabies was later confirmed by FA testing of brain tissue impression smears. The 2 treatment raccoons survived until termination of the experiment 48 days post-challenge. No clinical signs of rabies were observed and FA staining of brain tissue impression smears did not indicate any rabies antigen was present.

This study lacks the necessary monitoring and controls to state definitely that the raccoons were protected by the adoptive transfer of CMI; however, the challenge virus dose of $10^{5.3}$ MICLD50 is considered to be sufficiently high to give 100% mortality to the raccoons on test. Sikes and Tierkel (1960) found a 70% mortality rate in raccoons inoculated IM with $10^{4.2}$ MICLD50 of rabies virus of fox salivary gland origin, and McLean in 1975 (describing the unpublished data of Sanderson) reported a 66% mortality rate in raccoons inoculated with $10^{4.0}$ MICLD50 of rabies virus of skunk origin.

There is a remote possibility that sufficient time had not been allowed for the disease to manifest itself in the 2 surviving raccoons. In the Sikes and Tierkel (1960) and the Sanderson (McLean 1975) studies, incubation periods in raccoons challenged with $10^{4.2}$ to $10^{4.0}$ MICLD50 of rabies virus ranged from 13 to 35 days. However, 1 raccoon inoculated

with $10^{3.8}$ MICLD50 of rabies virus in the Sanderson study survived

for 107 days post-inoculation. This raccoon died of rabies but only

5 days after having been administered 12 daily injections of 10 mg of

cortisone acetate.

It has been suggested that leucocyte inhibition assays can be used

to detect TF activity in vitro (Wilson et al. 1979a,b and Borkowsky

and Lawrence 1979a,b). Levin et al. (1970) reported induction of migration

inhibition factor after injection of TF into patients with Wiskott-

Aldrich syndrome and Goust et al. (1976) reported the induction of

leucocyte migration inhibition factor after injection of TF into

patients with measles. In Trial B of the wildlife studies, in which

TF was administered to striped skunks, the ILMI assay was used to

determine if an increased cellular inactivity could be detected in vitro

2 days after treatment. As indicated in Table 16, 2 of 3 skunks treated

with TF preparation G became sensitized to rabies virus. This would

suggest that the TF treatment did specifically stimulate skunk

lymphocytes.

The lymphocytes from the 1 skunk treated with TF preparation H

apparently were not sensitized to rabies antigen and did not produce

migration inhibition factor detectable in the in vitro test. Lymphocytes

collected from control animals also remained negative for migration

inhibition factor.

None of the skunks inoculated IM with $10^{6.3}$ MICLD50 died of rabies

but rather all remained healthy until they were killed at the termination

of the experiment. However, 1 skunk (663) treated with TF preparation

G and 1 skunk (666) treated with TF preparation H died of rabies after challenge with $10^{6.6}$ MICLD50 CVS-27 rabies virus. Neither of these animals had a detectable cellular immune response prior to challenge. Although these findings may suggest that a rabies specific cellular response indicates protection from rabies infection, control animals which did not have detectable cellular responses also survived challenged with the virus. Alternative mechanisms of resistance may have influenced the protection observed. A very high intestinal parasite load was noted at necropsy of the 1 surviving control skunk challenged with the higher concentration of virus. Although parasites were observed in some other animals, the level of infection was much lower. Helminths have been shown to potentiate the immune response to unrelated antigens nonspecifically (Welch 1976).

The dose of challenge virus inoculum should have been sufficient to give 100% mortality in nonimmune animals. Sikes and Tierkel (1960) reported that a challenge dose of $10^{5.1}$ MICLD50 rabies street virus resulted in 100% mortality in striped skunks. The skunks in this study were inoculated with a laboratory strain of the virus (CVS-27) which had been propagated in mice for 5 passages. It is possible that the virulence of the virus was lower for the skunks than it was for mice. This phenomenon has since been observed in this laboratory during other studies with skunks that were inoculated with CVS-27 rabies virus propagated in mouse brain.

It is well-documented that TF does not augment the antibody production (Lawrence 1974), and it has been proposed that its activity is restricted

to a subpopulation of cytotoxic T-cells (Basten et al. 1975).
Theoretically, treatment with both vaccine (to stimulate the humoral
and cellular response) and TF (to stimulate the cellular response)
could have an additive effect. Assuming that this would be better than
immunization with either vaccine or TF alone, a higher survival rate
would be expected in mice treated with both agents. This theory was
tested in mice with inconclusive results. The 2 groups of mice treated
with vaccine had a higher survival rate than did those treated with
TF alone or the untreated controls. Since all mice treated with vaccine
alone or with vaccine plus TF survived challenge with rabies virus, no
distinction could be detected between the vaccinated groups. At the
termination of the study, antibody was detected in both vaccine-treated
groups of mice but not in the TF-treated mice nor in the untreated
controls. No significant differences in antibody titers were seen between
the 2 vaccinated groups of mice, supporting the thesis that TF has no
direct effect on the stimulation of the humoral immune system. Since
antibody was tested at 21 days post-challenge with rabies virus, it is
conceivable that, by this time, some mice treated with TF alone or
untreated mice could also have seroconverted. However, for unknown reasons,
no antibody was detected in sera from these groups of mice.

Recent in vitro (Wiktor et al. 1974, Hill 1974 and Wiktor et al.
1977a,b) and in vivo (Lagrange et al. 1978, Shope et al. 1979 and Tsiang
and Lagrange 1980) studies have indicated that the cellular immune
response contributes to an animal's ability to resist viral infections.
Wiktor et al. (1977b), using the chromium release assay, suggested that

the level of resistance is directly correlated with the level of cytotoxic T-cell response and Miller et al. (1978) reported that CMI was involved in the clearance of rabies virus from the CNS. Other studies have also suggested that the cellular immune response has a vital role in the development of the pathologic conditions responsible for death following rabies infections. Iwasaki et al. (1977) found that nude mice (a highly inbred strain of mice characterized by a lack of T-cell function) experienced a significantly longer mean survival time (14.0 days) than did immunocompetent mice (mean survival time 12.0 days) after experimental infection with rabies virus.

In mouse trials D and E described in this dissertation, treatment of mice with TF resulted in a reduced resistance to challenge with rabies virus. Treated mice in both trials not only had a higher mortality ratio than did untreated control mice, but also exhibited a shorter incubation period for the infection and a shorter duration of morbidity. These observations appear to be in direct conflict with the previously described positive results of adoptive immunization of mice, a guinea pig and the raccoons. However, many variables exist in the preliminary studies, including: 1) the source of the immunizing virus, 2) the species of animal immunized for preparation of TF, 3) the purification procedure for the TF preparations, 4) the dose of TF administered, 5) the species of animal adoptively immunized, 6) the route of administration of TF, 7) the schedule of immunization, and 8) the source of challenge virus. Any 1 or combination of the above variables may have influenced the

observed results. However, many of the studies did indicate that the activity of TF may regulate the ability of an animal to resist rabies viral infection.

Although the preliminary studies were designed to develop adoptive immunization techniques and to give an indication of the feasibility of using TF to immunize animals against rabies virus, the follow up series of experiments was designed to minimize the variability described above, to quantitate more effectively the adoptive immunization procedures, and to measure the results more accurately. These experiments were performed in vivo with the use of both inbred and outbred mice and in vitro with the use of mouse splenocytes. The in vitro and in vivo studies were performed with TF preparations from the same spleen cell populations, and both the in vitro and in vivo TF activities were monitored using the lymphocyte proliferation assay. The dosage of TF used in these studies was defined by the amount of RNA contained in the TF preparation as determined by the orcinol reaction.

Ascher et al. (1974) described studies with TF prepared by dialysis in which he was able to demonstrate the adoptive transfer of immunity to tuberculin, streptokinase-streptodolnase, diphtheria toxoid, and coccidioidin in vivo from human donors to human recipients and in vitro from immune lymphocytes to nonimmune lymphocytes. They reported that TF prepared by the conventional technique of dialysis of leucocyte extracts against distilled water was not satisfactory for in vitro use, but, when the leucocyte extracts were dialyzed against tissue culture medium, the activity of the TF for in vitro use was enhanced and could

be measured in vitro.

In an attempt to demonstrate rabies-specific activity of TF in vitro, spleen cells from nonimmune mice were cultured in the presence of various dilutions of antigen and various concentrations of TF derived from both immunized and nonimmunized mice. As seen in Table 17, no specific or nonspecific stimulation of the normal lymphocytes was detected. Stimulation of the same cell populations with Con-A indicated that the cells were capable of undergoing transformation.

In the Ascher et al. (1974) study, time-course experiments indicated that 7-day cell cultures were necessary to demonstrate maximal differences in cell proliferation caused by TF_{DM}. Hill (1974) reported a maximum proliferative response of mouse lymphocytes cultured in the presence of antigen for 4 days and a sharp fivefold reduction of the stimulation index by day 6. It is possible that the 7-day culture recommended by Ascher et al. (1974) for the stimulation of naive lymphocytes by TF in vitro and the recommended 4 day incubation period for rabies antigen stimulation of sensitive lymphocytes were mutually exclusive. As a result no stimulation was detectable. It is also possible that no adoptive transfer of immunity had occurred. Salaman (1978), in a study designed to repeat the Ascher et al. (1974) study, also was unable to demonstrate any antigen-specific transfer of sensitivity to normal lymphocytes in vitro.

When spleen cell lymphocytes from mice which had been inoculated with either TF_{DW+} or TF_{DW-} 2 days prior to harvest were cultured

without antigen, a significantly (P<.001) higher level of background

stimulation was observed. Salaman (1978) described a similar background

stimulation when lymphocytes from tuberculin negative persons were

cultured in the presence of TF also derived from tuberculin negative

donors. However, in the in vitro system of Salaman, no antigen-

specific proliferation was detected above the background nonspecific

stimulation.

The results outlined in Table 18 indicate that significantly higher

(P<.001) stimulation indices were noted in lymphocyte cultures from

mice which had been inoculated with TF from rabies positive mice and

cultured in the presence of rabies antigen than was noted in lymphocytes

from mice which had been administered either TF_{DW-} or saline. These

data strongly suggest that the TF_{DW+} was capable of specifically

transferring sensitivity to rabies virus in vivo.

No correlation between the specific and nonspecific stimulation

of lymphocytes and resistance to rabies infection was observed. Figure

7 indicated that mice treated with various dosages of TF_{DW+}, TF_{DW-},

or a saline placebo all had similar mortality patterns after challenge

with CVS-27 rabies virus. In addition, the mortality and the onset

of morbidity were similar in all treatment and control groups of mice,

in contrast to the observations noted earlier where treatment with

column-purified TF appeared to result in shorter incubation periods

and earlier mortality for the mice.

No definitive conclusions can be drawn as to the efficacy of TF

as an immunizing agent against rabies virus. Mortality data were

highly variable, however, it did appear that treatment with TF may have been an influence in the resistance to infection exhibited by some mice, a guinea pig, raccoons and some skunks. Resistance from rabies also appeared to have been inhibited in 2 of the trials done in mice and no effect was noted in other mouse trials.

Evidence of specific stimulation of CMI with TF used in vivo was demonstrated both in skunks and in mice using in vitro assays. However, data indicating a correlation between resistance to challenge are lacking and only questionable in the trial utilizing skunks. A high nonspecific stimulation of lymphocyte proliferation was observed when mice were treated with either TF_{DW+} or TF_{FW-}. Finally TF_{DM} did not, either specifically or nonspecifically, stimulate naive lymphocytes in vitro.

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APPENDIX

Raccoon, Fox, Bat, and Skunk Rabies in the United States

Raccoon rabies

Raccoons are broadly distributed in the continental United States with the exception of isolated areas in the high mountain and desert regions of the West (Schwartz and Schwartz 1959). Rabies in this species occurred infrequently in most areas but was endemic in Georgia, Florida, Alabama and South Carolina (Scatterday et al. 1960, Irvin 1970, Prather et al. 1975 and CDC 1980g). In 1979, 93% of 543 cases of raccoon rabies found in the United States were reported from these four states.

Sikes and Tierkel (1960) found raccoons to be approximately 1000 times more resistant to rabies of fox salivary gland origin than were striped skunks. They observed comparatively shorter incubation periods and longer morbidity periods in raccoons than in foxes and in skunks. McLean (1975), reporting unpublished data of G. C. Sanderson, identified a somewhat longer incubation period (ranging from 18 to 66 days) in raccoons inoculated with rabies virus isolated from raccoon salivary glands than the 10 to 42 days incubation period seen in the raccoon of the Sikes and Tierkel (1960) study. One unusually long incubation period was observed in a raccoon (in the Sanderson study) which died of rabies 107 days after inoculation with the virus and 3 days after the completion of 12 daily injections of 10 mg of cortisone acetate. No clinical signs of rabies were observed prior to the initiation of cortisone treatments. Bigler et al. (1973) reported 2 naturally infected raccoons died of rabies 30 and 70 days after capture. Since the time and the source of exposure cound not be determined, these suggested a minimum incubation period.

Scatterday et al. (1960) described the behavior of 38 naturally infected rabid raccoons in Florida as totally unaggressive. No unprovoked attacks were reported. Similar observations were made after another Florida study by Kappus et al. (1970) who identified only 1 of 31 rabid faccoons as frankly aggressive. Sick or peculiar behavior was the most frequently reported characteristic.

However, more recent studies have indicated that aggressive behavior is not uncommon in rabid raccoons. Of 570 rabid raccoons in Georgia, 47% were reported as showing aggressive behavior: 39% were found within yards during daylight; 34% were fighting with dogs; 13% showed no fear when attacking human beings; 7% were walking with an irregular gait; and 6% were killed or found dead on the road (McLean 1975, reporting unpublished data of Dreesen and Burnham). McLean (1975) further claimed that 46% of rabid raccoons from Florida and Georgia displayed some sort of aggressive behavior.

McLean (1975) suggested that raccoons pose a greater threat for human exposure than do other wildlife species. Raccoons readily adapt to life in urban situations where they not only are tolerated, but often are treated as semi-domestic pets (Bigler et al. 1973). They often bite or scratch people who, thinking they are tame, approach them (McLean 1971). Furthermore, a relatively large proportion of raccoons may become infected in epidemics. During a rabies epidemic in Florida, Kappus et al. (1970) found that 10% of nonrabies-suspect raccoons were positive for the virus both in their saliva and in their brain tissues.

The potential hazard of exposure to rabies by raccoons kept as

pets was emphasized recently in Florida (CDC 1980c). Public health officials determined that at least 150 people had been exposed to a rabid pet raccoon. Rabies post-exposure prophylaxis at an estimated minimal cost of over $20,000 was recommended for 74 of those exposed.

Fox rabies

Rabies in foxes in the United States has been recognized for more than 200 years. The disease apparently was introduced into this country by imported dogs from which it spread to foxes (Steele 1975). The first major epidemic was reported by Thatcher in 1812 in Massachusetts (Gier 1948). Since that time epidemics of fox rabies have occurred primarily east of the Mississippi River (Wilkison 1893-94, Guilford 1938, Schoening 1942, Compton 1943, Garlough 1945, Johnson 1945, Wood 1954, Parker et al. 1957, Friend 1968 and Sikes 1975).

Three species of foxes are of primary importance in the epidemiology of sylvatic rabies in the United States. The red fox (Vulpes vulpes) is broadly distributed from the Arctic to south Texas with the exception of the extreme southeast and the arid regions of the middle and far west. The gray fox (Urocyon cinereoargenteus) occurs in broad areas of the 48 contiguous states. It is not found in much of the northern 2 tiers of states from Washington to North Dakota or in western Nebraska and Kansas. A third species, the Arctic fox (Alopex lagopus), is restricted in its distribution to the tundra zone of northern and western Alaskan islands in the Bering Sea and has been introduced onto many of the Aleutian Islands (Rausch 1958).

Generally, in reports of the incidence of fox rabies in the United States, no distinction is made among the various species of foxes. However, from isolated surveys, it can be seen that red fox rabies is most prevalent in the northeast, whereas gray fox rabies is epidemiologically important in southeastern states (Sikes 1975). Parker et al. (1957), Friend (1968) and Schnurrenberger et al. (1970b) have reported that during epidemics of fox rabies the ratio between reported cases in the 2 species was approximately equal to the ratio of their population size as determined from trapping records. No significant difference between red and gray foxes in the prevalence of rabies was reported by Davis and Wood (1959) in Georgia, Florida, and South Carolina. Rausch (1958) made a similar observation when discussing the disease in relation to the species distribution of Arctic and red foxes in Alaska.

Nationally, reported cases of rabies in foxes have declined in the 25 year period between 1953 and 1977 from over 1000 cases in 1953 to 122 cases in 1977. Whereas foxes represented 70% of all sylvatic rabies reported in 1953, they accounted for only 3% in 1979 (CDC 1978b and 1980a). In 1979, only Maine and Alaska reported more cases of rabies in foxes than in any other species (CDC 1980a).

Foxes are highly susceptible to rabies viral infection (Sikes 1962). Although incubation periods of as long as 13 months have been reported for rabies in foxes (Steele 1967), this is probably not usual. Sikes (1962) reported that the disease seen in foxes inoculated with what is considered to be a large inoculum of virus (10^3 MICLD50)

had a short incubation period (less than 18 days); smaller inocula resulted in incubation periods of 38 days or more. Titers of 10^3 MICLD50 virus or greater were seen in the salivary gland of foxes with clinical disease appearing after long incubation periods. Based on the failure to isolate rabies virus from salivary glands of 262 foxes without concurrent infection of the CNS, Tierkel (1959) concluded that the fox is unable to transmit the disease as a symptomless carrier.

Attempts to control fox rabies have concentrated on the reduction of fox populations in epidemic areas. In central Europe, the reduction of fox numbers by hunting and gassing of dens has been shown to be a valuable approach in the control of fox rabies (Wandeler et al. 1974a, b). In the United States, however, the efficacy of using population reduction as a technique to control fox rabies is inconsistent. In New York, the reduction of fox abundance appears to have reduced the incidence of rabies in some areas of the state and intensive trapping has virtually eliminated rabies in certain areas (Linhart 1960). Virginia instituted a predator control program in 1961 in an effort to control sylvatic rabies. By 1969, the advisability of continuing the program was seriously questioned (Carey et al. 1978). They suggested that population reduction for the control of rabies may be detrimental instead of beneficial because this practice allows a younger, more susceptible population to become established. It was estimated that 70 to 80% of the animals would have to be removed to result in a reduced population in a successive year. With the immigration of young animals

from surrounding areas, the effects of even severe population reduction would be negated. The difficulty involved in the removal of sufficient numbers of foxes is magnified by the high cost of removal. Estimated costs of $25 to $200 per animal trapped have been reported (Linhart 1960, Lewis 1968 and Parks 1968).

It is estimated that a fox population density of greater than 1 fox per square kilometer is necessary for the establishment of epidemic fox rabies (Anon. 1973 and Dean 1955). However, Friend (1968) and Carey et al. (1978) suggested that some ecologic barriers can cause exceptions to the rule. Parker et al. (1957) found that significantly higher fox population densities existed in rabies-free areas of New York than existed in rabies-enzootic areas. A similar observation was made by Davis and Wood (1959) in Georgia, Florida, and South Carolina.

Bat rabies

Bat rabies was first diagnosed in the United States in a Florida yellow bat (_Dasypterus floridanus_) in 1953 (Venters et al. 1954). Since that time rabies in bats has been reported in every state of the United States except Alaska and Hawaii. Cases of rabies in bats reported to CDC are geographically more widely distributed than cases in any other animal host (CDC 1977a). The incidence of bat rabies has steadily increased from 8 cases in 1953 to 756 cases in 1979 (CDC 1980a). In the United States 10 cases of human rabies have been attributed to exposure to rabid bats (CDC 1977a).

Rabies virus has been identified in specimens from 30 of the 39

recognized native species of bats (Constantine 1979a). In the United

States chiropteran rabies is limited to insectivorous bats, but in

Latin America vampire bats, in the family of Desmodontidae, are

responsible for hundreds of human deaths and have served as a major

limiting factor to the livestock industry (Acha 1966 and Bisseru 1972).

The big brown bat (Eptesicus fuscus), the Mexican free-tailed bat

(Tadarida brasiliensis mexicana), and various species of the genus

Myotis are the bat species most frequently reported to be infected in

the United States (Bigler et al. 1975).

Although the ultimate importance of bats as vectors of rabies

is unknown, in terms of both numbers and geographic distribution they

constitute one of the largest potential reservoirs of the virus in

the United States today. The majority of the species are gregarious,

living in colonies of hundreds, sometimes millions, in dark confined

areas. Other species live as solitary individuals in more open areas

in tree foliage (Constantine 1979b).

Infected bats rarely attack man but many persons and pets are

bitten each year when they handle sick bats (Constantine 1979b). If bats

prove to be a source of rabies for wild carnivores, they may be a greater

public health hazard than we realize (Constantine 1979b).

Another epidemiologically important characteristic of rabies

infections in bats is the apparent capacity for bats to become latently

infected and act as carriers of the virus for several months (Burns

et al. 1956, Sulkin 1962 and Constantine 1971).

Constantine (1967) theorized that there are two distinct syndromes

of species-specific bat rabies--one that does not kill the bat and
another one that does. He suggested that the nonlethal infection,
in which neutralizing antibody generally develops, in the absence of
clinical signs of rabies, is typical of the disease in gregarious bats.
Occasionally paralysis is observed. He speculated that viral trans-
mission occurs in the course of normal behavior, possibly by aerosol
or by casual biting (Constantine 1971). Gough and Jorgenson (unpublished
data; Veterinary Medical Research Institute, Iowa State University) found
evidence of transplacental infection in big brown bats in Iowa. One
female bat delivered two young after capture. Rabies viral antigen was
identified by specific FA antibody examination of impression smears of
brain tissue of both the mother bat and her 2 infants. The brain of a
second female captured from the same colony and 2 fetuses removed from
her uterus also were positive for rabies antigen by FA staining. Sims
and Sulkin (1963) reported similar findings in fetuses of experimentally
infected Mexican free-tailed bats.

The lethal rabies infection in bats occurs more frequently among
solitary bats. These bats develop furious rabies, attack without
provocation, and die with encephalitis. This syndrome can be perpe-
trated only if the virus incites these solitary bats to deliberately
seek out and attack others of their kind (Constantine 1971).

There is believed to be no strong causal relationship between
rabies in bats and in rabies in terrestrial animals (Stamm et al.
1956 and Winkler 1972). However, Winkler (1972) suggested that in
unique situations where rabid bats are in abnormally close association

with other susceptible species, the bats may serve as a reservoir of virus for terrestrial animals. Twente (1955) reported heavy predation on bats by raccoons in Kansas gypsum caverns, and Frederickson and Thomas (1965) have suggested a relationship between rabies in bats and in foxes in caves in Tennessee.

Constantine, in a series of publications, has reported that carnivorous wildlife can be infected with rabies virus after being bitten by rabid bats (Constantine 1966c, Constantine and Woodall 1966 and Constantine et al. 1968), after IM inoculation of isolates of bat rabies virus (Constantine 1966 a,b, Constantine and Woodall 1966 and Constantine et al. 1968).and by nonbite routes after being exposed to the atmosphere of caves inhabited by bats (Constantine 1962). Winkler (1968) was able to isolate rabies virus from a bat cave with the use of a mechanical air sampler.

Rabies virus has been isolated from brain, salivary glands, mammary glands, and intrascapular brown adipose tissue of bats (Sulkin et al. 1959, Sulkin 1962 and Sims and Sulkin 1963). Evidence to suggest that viral storage and replication occur in the intrascapular brown fat was provided by Sulkin et al. (1959) and Sims and Sulkin (1963). This issue is an actively metabolizing source of nutrition during periods of hibernation or torpor (Wertheimer and Shapiro 1948 and Remillard 1958). Rabies virus was detected in the brown fat of 20 of 20 Mexican free-tailed bats inoculated with 8000 MICLD50 of rabies virus. Viral titers in brown fat ranged from 10 to 1000 MICLD50s (Sims and Sulkin 1963).

Bat rabies is most frequently reported in areas of dense human populations. This phenomenon can be attributed to a greater potential for the infected bat to be observed and subsequently submitted for diagnosis of rabies. Constantine (1979b) has found that less than 0.05% of free ranging bats are harboring the virus. He suggested that bites usually occur when curious people handle sick bats and that the contact between the public and bats must be prevented. He suggested that the frequency of exposure can best be lowered by warning the public against handling sick bats and correcting deficiencies in construction of buildings that make them accessible to bats.

Skunk rabies

Skunks are the principal wildlife reservoir for rabies in the United States. Two species of skunks are of primary importance but, as in reporting of fox rabies, little effort has been made to distinguish between the skunk species involved. In 1959, Johnson speculated that the spotted skunk (Spilogale putorius) was the reservoir for rabies in the United States and that the striped skunk (Mephitis mephitis) represented an epidemic indicator species. This theory has not been widely accepted but rather it is generally believed that the virus is maintained and transmitted by striped skunks in broad areas of the United States (Friend 1968 and Houseknecht 1969). Currently, more cases of rabies in striped skunks are reported than in any other animal species.

The spotted skunk is found in broad areas of the contiguous United States. It is generally absent from the northern tier of the states

west of the Mississippi River, the northern two tiers of states east

of the Mississippi River, and the eastern coastal states except for

Florida. Striped skunks are found throughout the United States

(Schwartz and Schwartz 1959).

It appears that the spotted skunk was important in the dissem-

ination of the disease westward during the early years of the history

of the disease in wildlife in the United States. The earliest report

of skunk rabies in the United States was in 1826 by Duhaut-Cilly who

noted that spotted skunks sometimes entered houses at night, bit people,

and gave them hydrophobia (Johnson 1959). Johnson (1959) wrote, "The

human cases of rabies were, for the most part, the result of bites by

the spotted skunk and this is why this animal is known as the 'phoby

cat' in the Southwest."

The current epizootic of skunk rabies appears to have started in

the early 1950's (Parker 1961). In 1953, there were 319 cases of skunk

rabies reported in the United States. This made up 4% of the total

rabies cases reported and 22% of all the wildlife rabies reported for

that year. In 1979, skunks represented 59% of 5,119 total cases reported

and 68% of all wildlife rabies reported (CDC 1980a). It can be argued

that the apparent increased prevalence of skunk rabies is a statistical

aberration of awareness and reporting, but, while skunk rabies was

reported 9.5 times more frequently in 1979 than in 1953, fox rabies

was reported 7.4 times less often (CDC 1978b and 1980a). Since the

reporting of rabies in these 2 species would have similar limitations,

these figures give some measure of reality to the increase in incidence

of skunk rabies in the United States. Factors such as changes in population levels and habitat preferences may be involved in the apparent shift in relative importance between foxes and skunks.

Rabid skunks exhibit a broad range of behavioral patterns from overtly furious to unusually friendly and docile. While observing an epidemic of rabies in a captive skunk colony, Gough and Niemeyer (1975) noted that rabid skunks were frequently found dead without any evident clinical signs of the disease having been noted. In an Ohio study of 95 rabid skunks, 35 instances of lack of coordination and staggering, 37 instances of viciousness and 25 instances of friendliness were noted. Only 5 skunks were reported to be foaming at the mouth (Schnurrenbeger et al. 1964). In observations of 473 rabid skunks in Illinois, Schnurrenbeger et al. (1970a) determined the predominant clinical signs to be movements during the day time, agressiveness, lack of fear, and incoordination. In both the Ohio and the Illinois studies, skunks frequently were reported to be attacking dogs.

Storm and Verts (1966) observed the movements of a naturally infected rabid skunk during a radiotelemetric study of skunks in Illinois. The animal was apparently normal when the transmitter was attached but, when she failed to move for 4 days from an underground den, the den was excavated and she was found dead. Thirty-two days had elapsed from the date the transmitter was attached until she was found dead. Rabies virus was isolated from the skunk's brain and salivary gland by mouse inoculation and was identified by the FA technique. The skunk had not traveled in a pattern overtly different

than that of four nonrabid skunks which also had been equipped with radiotransmitters. This is consistent with other reports of movements of rabid skunks (Schnurrenberger et al. 1964 and Schnurrenberger et al. 1970a).

Although rabies in skunks is reported throughout the year, generally the majority of cases occur from early spring through midsummer (Friend 1968 and Schnurrenberger et al. 1970a). Secondary peaks of skunk rabies are noted in the fall when population densities are at a maximum (Friend 1968). Verts (1967) suggested a close correlation between the spring and summer peak and the breeding and parturition of striped skunks. Communal denning of striped skunks in the winter has been suggested as another possible factor in the spread of the disease (Parker 1961 and Houseknecht 1969).

The nematode sinus worm (Skrjabingylus spp) was considered as a potential mode of transmission for rabies virus in skunks. Investigations into the transmission of the virus throughout its life cycle, however, indicated that the worm was not involved (Ewing and Hibbs 1966 and Emmons and Johnson 1969). Beauregard and Casey (1973) ruled out the possibility of transmission of rabies by way of the skunk scent glands.

In experimental infections of skunks with rabies virus of fox salivary gland origin, Sikes and Tierkel (1960) found that a virus titer of 1.4 x 10^3 MICLD50 was necessary to induce fatal disease in skunks inoculated intramuscularly. The incubation period varied from 17 to 88 days. Clinical illness was observed in the skunks for 1-13

days. They found that 15 of 18 skunks which died of rabies had detectable virus in their saliva and 3 of these had almost 10^6 MICLD50 per 0.03 ml.

Rabies in skunks in the United States presents a major public health hazard. Although direct transmission of rabies virus from wild skunks to man is limited, indirect transmission of the virus to man via domestic livestock or pets is a major concern. In Iowa, there is a close association between skunks and cattle both on pasture and in feed lots. Beran (unpublished data, 1975; College of Veterinary Medicine, Iowa State University) reported that in Iowa at least 54 persons underwent rabies prophylactic treatment as a result of exposure to rabid cattle for the 2 year period of 1974-1975. Although skunks are probably the primary vector for rabies in cattle in Iowa, on a world wide basis, there are only 13 reported cases of human rabies as a result of exposure to rabid cattle. Most of the cases could be traced back to rabid foxes and none were the result of skunks transmitting rabies to cattle (CDC 1980f).

Striped skunks are considered by many people to be interesting and affectionate pets. However, an increasing number of cases of rabies are being reported in wild pets, especially in skunks. In the 3 year period of 1977 through 1979, CDC reported that 151 persons were exposed to a total of 9 rabid pet skunks in Oklahoma, Montana, Indiana, Arizona and Oregon (CDC 1979b). Five persons in New Jersey underwent antirabies treatment after exposure to a pet skunk suspected of being rabid (Debbie 1979). The sale of and the keeping of skunks as pets is strongly discouraged because they are potential sources of rabies.

Little direct effort is made to control skunks. Removal of garbage and debris around farm buildings and the burning of fence rows are possible ways of discouraging habitation by skunks. Indirect control of populations occurs when skunks are killed after they are caught in traps set for fox, coyote, and raccoon during the trapping season. Many more die as a result of highway traffic.