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A PRIMARY GOAT KIDNEY CELL RABIES VACCINE FOR HUMAN USE

by

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The goat kidney rabies vaccine was investigated as a substitute for the nervous tissue rabies vaccine. Kissling Rabies virus (fixed strain) provided by Michigan State Department of Public Health Laboratories was grown in primary goat kidney cells. Results showed that the virus does not need adaptation to goat kidney cells. Rabies vaccine was prepared by adsorption on aluminum phosphate. The potency of the vaccine determined by the standard NIH mouse potency test meets the requirements of the World Health Organization. Pre-exposure immunizations of guinea pigs resulted in satisfactory antibody levels and post-exposure treatment regimes of guinea pigs challenged with street virus confirmed significant protection.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Rabies is a viral disease that affects all hot blooded animals and is carried to other animals and man through different ways. It has a quite, different pattern from the more familiar viral diseases like measles, polio and mumps. With these infections the virus spreads via the bloodstream to the lymph glands where the virus multiplies and spreads to the target organ of the particular disease. In all these diseases, the defense mechanisms of the body go into action at once, to prevent multiplication of the virus and in the end to destroy it and clear it out of the body. Protection by vaccination stimulates the system to produce its own antibodies to the disease and is comparatively easy to establish against viral diseases like smallpox and polio.

Rabies develop in a quite different way. It has an extremely long incubation period, which varies from case to case, followed by a progressive inflammation and breaking down of the central nervous system. When the disease spreads via the nerve cells to become concentrated in the area of the brain known as Ammon's horn, where it destroys the cells it infects. This area of the brain, which runs across the middle of the head, from side to side, controls sensations of the tongue, face, neck and movements of the legs, body, arms as well as speech. When a sufficient number of brain cells have been destroyed so that the patient's use of the brain's function is impaired, the obvious signs and symptoms of rabies occur. One advantage we have against rabies is that the long incubation period allows steps to be taken to prevent the disease developing after a person has been exposed to rabies, this is not possible in the more common viral diseases like

influenza or measles. Antirabies vaccine can be given to stimulate production of antibodies before the virus starts to multiply and move from the site of the bite into the nervous system.

A satisfactory vaccine has to meet certain requirements, which include safety, potency, ease of production and stability even after a prolonged storage period.

With increasing emphasis on procedures aimed at increasing the safety of the product there has been a tendency for potencies to drop. This is seen in the duck-embryo vaccine where the small amount of nervous tissue used greatly reduces the hazard of postvaccinal complications of the central nervous system, but the immunogenic potency is usually somewhat less than that of the brain tissue vaccine.

So far production studies indicate that rabies viruses throughout the world and from a variety of species have the same general antigenic structure, so there is no particular reason to use a local strain of virus in vaccine production. Also there is a great advantage in using established standard strains, because extensive experience of laboratory production and field use, which is already done on these strains, is very difficult to obtain with newly developed strains. In general, a virus for vaccine production should come from a frozen working pool kept for long-term use. The virus should not be passed many times or in different species between removals of portions to serve as innoculum for vaccine production.

In the case of vaccines prepared from virus grown in animals, the quality of the animals is very important. Animals should be from controlled stock, in good health, and free from known latent infections. For vaccines prepared from the brain of suckling animals, the age of the animal at the time the brain is harvested is the determining factor for the presence of the material responsible for allergic

encephalitis reactions in the central nervous system tissue. Therefore, using older animals for vaccine production must be avoided.

Propagation of rabies virus in primary cell culture systems and adaptation to several established cell lines has made a systematized approach to the study of rabies virus and its interaction with host cells possible.

Preservation of potency on prolonged storage can be achieved by lyophilization. This is possible with vaccines inactivated by a removable agent as an example of ultraviolet irradiation and β-propiolactone).

An ideal vaccine would be prepared from virus source material containing no foreign proteins, would be easily and inexpensively produced in large volumes with uniformly high titers, and would be inactivated by a method that consistently retained high immunizing potencies. Furthermore, it would be capable of long-term storage without a decrease in potency, would cause no reactions on repeated innoculation of patients, and would be effective with fewer doses.

In general, a method that gives a safe and quantitatively proven potent vaccine is desirable. Since none of the vaccines at present available can meet all the requirements, research will continue for vaccine improvement.

OBJECTIVE

Fermi type vaccine is a vaccine composed of rabies virus-infected nervous tissue sterilized by phenol. The conditions employed in the preparation of Fermi vaccine (5% brain tissue incubated in 0.5-1% phenol at 22-24°C) do not totally inactivate the virus. The vaccine is given in successive doses for 14-21 days. Each successive innoculation consists of an identical dose normally 5-6 ml^3 of the same vaccine preparation. This is the only live virus, nervous tissue, rabies vaccine still in use to a significant extent, primarily in northern Africa.

Because of natural fear of the dangers of innoculating any living rabies virus into man, and the complication that might occur in the central nervous system due to the use of brain tissue vaccine, this study was initiated as a trial to prepare an inactivated rabies virus vaccine propagated in primary goat kidney cells, hoping for a safe, potent, easily prepared and inexpensive product, to use as a substitute for Fermi-type rabies vaccine. Therefore, the objectives of the study were:

1. Preparation of the primary culture of the goat kidney cell.
2. Examination of virus adaptation and propagation.
3. Examination of virus inactivation, antigen concentration and absorbtion.
4. Conducting pre-exposure and post-exposure experiments on animals.

LITERATURE REVIEW

Rabies is essentially a virus disease of animals, which is transferred to man and other animals by means of a bite, a lick on a sore place, through the mucous membranes of mouth and eyes, or by breathing the virus particles from the air in the case of transference from vampire bat, where they have been present in great concentration. Any warm-blooded animal, from a mouse or an elephant to a man may contract rabies, but some species, those which are normally "biters", are more prone to pass the disease on.

The very names by which the disease is known strike terrors. Rabies is from the latin word Rabere, to rave indicating the deranged mental state of the patient; hydrophobia, the fear of water which is one of the major signs of the disease in humans; Lyssa, the Greek word for frenzy, denoting the manic convulsions of brain fever induced by the virus. The rabies virus is classified as one of the rhabdovirus group, the name taken from the Greek term for rod-shaped.

History of Rabies

Rabies has a long and interesting history. Perhaps the earliest reference to rabies is that which occurs in the pre-Mosaic Eshnunna Code [which predates the better known code of Hammurabi of ancient Babylon in the twenty-third century B.C.]. In this code the following excerpt is found: "If a dog is mad the authorities have brought the fact to the knowledge of its owner; if he does not keep it in, it bites a man and causes his death, then the owner shall pay two-thirds

of a mina (40 shekels) of silver" (Tierkel, 1971). In the fourth century B.C., Democritus wrote a description of canine rabies in the natural history of animals, Book 8, Chapter 22, "that dogs suffer from the madness, this causes them to become very irritable and all animals they bite become diseased".

In the first century, a Roman physician, Celsus, studied rabies. He was emphatic that the bites of all animals that contained virus were dangerous to man and beast. He wrote to describe the disease, "the greeks call it hydrophoan, a most wretched disease, in which the sick person is tormented at the same time with thirst and the fear of water, and in which there is but little hope" (Smithcors, 1958). As a treatment he recommended the use of hot and cold baths. He stated that when disease appears, "the only remedy is to throw the patient unexpectedly into a pond, and if he has not a knowledge of swimming to allow him to sink, in order that he may drink, and to raise and again depress him, so that though unwillingly, he may be satisfied with water; for thus at the same time both the thirst and dread of water is removed." This formidable treatment was continued until the nineteenth century according to Fleming who cites that a cooper of Ghent being cured by submersion in the sea. He was dropped in the ocean from a ship and allowed to sink with the aid of irons. After a couple of submersions he was brought to the deck and revived, and lived (Fleming, 1872).

In the third century, treatment of lower animals was described by Vegetium Renatus, one of the early writers on veterinary medicine. He recommended as an antidote for cattle that have been bitten by a mad dog, to give the cattle the boiled liver of the dog to eat, or to make it into balls and force it down as medicine. Also, by that time an accurate description of the dog disease was written.

At the beginning of the ninth century, a Syrian doctor Jahiah-Ebn-Serapion, believed that the disease produced by the bite of a mad dog was incurable; though

he proposed that the patient should be made to swallow water by enclosing it in a globule of concrete honey, which was to be put in the mouth (Fleming, 1872).

Hydrophobia was mentioned by Rhazes, an Arab physician, who says that a certain man barked by night like a dog and died. He also described a patient who, when he beheld water, was seized with trembling and rigors, but when the water was removed the symptoms ceased (Fleming, 1872).

In the eleventh century the Arab physician, Ebncenna, speaks of rabies. He directs that the wound be kept open for 40 days and that ordinary blisters be placed on it. He alludes that hydrophobous persons bark like dogs and that they have a desire to bite people; that patients who attempted to drink suffocated, and the illness terminated in apoplexy. His observations altogether mark a step forward in understanding the disease. He describes a dull redness, or erythema, which had been designated rabic roseola. From this period the literature of rabies gradually expands and with the progress of medicine the remarks became more valuable and comprehensive.

Up until the middle ages, epizootics (outbreaks) were rare, most cases were singular bites of rabid dogs, and occasionally of wolfs, badgers, foxes and even bears. In the nineteenth century, rabies appears to have become more widespread in Europe, especially in France, Germany and England. An extensive outbreak in foxes occurred in eastern France beginning in 1803 (Smithcors, 1958). This was reported as the largest outbreak that had ever been seen.

During 1881, Pasteur published his first report on rabies a period when many scientists were attempting to transmit rabies from man to animals and from animals to animals. As a result of the experiments, he found out that the disease can be produced by injecting brain material from rabid animals directly into the brains of dogs, and the incubation period was shortened to 1 or 2 weeks, or at the most 3 weeks and this was an important advance in experimental rabies studies.

He also found that an animal which recovered after early symptoms of rabies was immune to later incubations, and that some dogs seemed to have a natural resistance. One of the most important things that Pasteur discussed in his report is that dealing primarily with the virus attenuation. If one passed rabies virus from dog to monkey and then from monkey to monkey the virulance of the virus fell off at each passage. If the virus was then returned to a dog, rabbit, or guinea pig it remained attenuated. By using a series of injections of attenuated virus, he made dogs immune or at least refractory. In 1886 Pasteur reported his results of treatment of 350 cases. Only one person developed rabies, since then Pasteur concluded that "the prophylaxis of rabies is established and it is time to create a center for vaccination against rabies". Since then the Pasteur Institute was founded for the treatment of both the French and foreign people. Within a decade there were Pasteur institutes throughout the world (Steel, 1975).

About this time there was considerable discussion as to the route whereby rabies virus reaches the nervous system: (1) by the bloodstream and (2) by the nerves. Clinical and experimental evidence favoring the theory that the virus progresses along the nerves supplying the region in which the virus first appeared were done by Diverstea and Zagari (1889). They showed that inoculation of fixed virus into the sciatic nerve of a rabbit and a dog caused death with the same symptoms as those of intracerebral inoculation. Furthermore, death could be prevented by cutting the nerve after injection. It was found that virus injected under the skin of Swiss mice is first detected 5 or 6 days later in the central nervous system at the site in direct connection with the inoculated area. This finding agreed with the idea that the virus does travel by way of the nerves (reviewed by Webster, 1942).

In studying the causal agent of rabies, Pasteur had speculated that the agent was a tiny one, unlike ordinary bacteria. In 1903 Negri, an Italian physician

discovered the bodies which bear this name, Negri referred to them as protozoa. These alleged parasites that Negri observed were seen especially in dogs, and were located in the horn of Ammon, especially in the larger nerve cells. They are also found in the spinal ganglia and in the spinal cord of dogs in which rabies occurred naturally or experimentally. Negri bodies were stained best with eosin methylene blue. They vary in size from 1 μm up to 10 or 15 μm or even as large as 27 μm by 5 μm. Galloway and Elford (1936) found that rabies virus falls among the largest viruses. They were the first to report on the size of the rabies virus.

THE HISTORY OF MAN VACCINATION AGAINST RABIES

A. Pasteur Treatment

Pasteur developed his antirabies immunoprophylaxis experimentally on dogs, and it became very famous after application to man. The virus strain used was isolated from the brain of a rabid cow in 1882. Pasteur established a strain of virus by a series of passages in rabbits, intracerebrally, the incubation period was "fixed" at 6-7 days. The virus was further attenuated by desiccation at room temperature over potassium hydroxide. The virulence of the cord tissue suspensions was found to decrease rapidly with successive days of dessication. It was demonstrated that dogs vaccinated with a series of 10 daily injections of cord suspensions of increasing virulence subsequently resisted challenge with virulent rabies virus, even when inculated intracerebrally. The requirement for a supply of fresh infected cords was resolved by the use of dessicated spinal cord after being preserved in glycerine. It has been demonstratred that dessicated cords preserved in glycerine retained the appropriate degree of virulence for a prolonged period of time (reviwed by Webster, 1942). The original Pasteur vaccination method has not been used in man since 1953, but vaccination schedules based upon those first described are still very much in use (Clark et al., 1975).

B. Nervous Tissue Vaccines

1. Living viruses

An early modifiication of the Pasteur method was described in 1887 by employing different degrees of dilutions rather than dessication to reduce the

virulence of the infected spinal cord preparations. This technique has the advantage of incubating a greatly decreased dose of nervous tissue, thus reducing the incidence of paralytic accidents. Fermi in 1908 recommended a vaccine composed of rabies virus-infected nervous tissue treated with phenol, this method did not inactivate the virus totally. Ether was also used to inactive the virus and an ether-inactivated vaccine was suggested in 1925 (Bisseru, 1972a).

2. Inactivated virus

The phenol-inactivated vaccine described by Semple (1911) has found by far the widest application, continuing to this day. It is an inactivated vaccine produced from adult animal nervous tissue. Inactivation of infected nervous tissue by ultraviolet (UV) irradiation was described by Hodes et al. (1940). It has been believed to yield vaccines of potency which is consistently equal to or superior to that of phenol-inactivated preparation (Habel, 1966b).

Attempts have been made to prepare vaccine from rabies virus-infected nervous tissue harvested from rodents under 10 days old which do not contain the central nervous system allergens responsible for paralytic incidents because of the continuous risk of inducing neuroparalytic accidents due to the use of adult animals (fully myelinated nervous tissue).

Fuenzlida and Palacios (1955) described a vaccine prepared from suckling mouse brain. The vaccine is 1% suspension of fixed virus-infected brains of suckling mice (harvested prior to 10 days of age) inactivated by UV irradiation or β-propiolactone. It is widely used for human antirabies treatment in Latin America (Fuenzalida et al., 1965).

Gispen et al. (1965) described a rabies vaccine of suckling rabbit brain origin, inactivated by UV irradiation, and it has been shown to consistently induce rabies antibodies in man .

C. Non-nervous Tissue Vaccine

Due to the side effects of using brain tissue from adult animals, the development of most new vaccines has been based upon attempts to avoid nervous tissue substrate altogether.

Dawson (1939), Klinger and Bernkopf (1939), two groups worked separately and succeeded at the same time to grow rabies virus in the developing chick embryos, they pointed out that the chick embryo virus after many passages showed greatly reduced virulence to rabbits. Later Koprowski and Cox (1948) adapted these methods for the production of a chick embryo origin vaccine.

A duck embryo vaccine which was widely used in the United States is prepared from a strain of fixed virus adapted to growth in 7-day old embryonated duck eggs (Powell and Culbertson, 1950). Embryos were harvested after 14 days. Vaccine is prepared by β-propiolactone inactivation of 10% infected embryo suspensions (Peck et al., 1956). Duck embryo vaccine has been demonstrated to contain very little encephalitogenic activity, but the high content of embryonic tissue frequently leads to minor allergic reactions to the assorted avian antigen present (MacFarlane and Culbertson, 1954).

Koprowski (1954) developed a vaccine of avian embryo origin it is the Flury high egg passage (HEP) vaccine. The vaccine is a suspension of chick embryo tissue infected with a human rabies isolate, highly attenuated by 136 passages in one day old chicks followed by approximately 180 passages in embryonated chick eggs. It has undergone several trials in man. Despite the fact that it contains living virus, studies by Fox et al. (1957) showed that the Flury HEP vaccine generally induces lower antibody titers in man than does the inactivated duck embryo vaccine, leading to the conclusion that Flury HEP is incapable of multiplying in man.

Tissue culture vaccine

Tissue culture vaccine goes back to 1936 when Webster and Clow were the first to propagate rabies virus in tissue culture, without loss of infectivity. It is an important method for the preparation of homogeneous cloned populations of the virus, which after concentration and purification yield higher titers of pure virus than can be obtained from the brains of infected animals, and it can be used for large scale production of vaccine. It is also important for understanding the basic structure of the virion.

The first rabies vaccine of cell culture origin extensively tested in man was developed in 1966 by Selimov and Aksenova. The SAD strain of fixed rabies virus was adapted to Syrian hamster kidney cell culture and then propagated in sheep embryo kidney cell cultures for vaccine production. The virus was attenuated with phenol (0.25%) at 22°C for 24 hours and lyophilized.

The adaptation of rabies virus to several established cell lines and strains has made possible a systematized approach to the study of rabies virus and its interaction with host cells. Baby hamster kidney (BHk/21) cells have become the favorite substrate for rabies virus investigations in vitro because of their extreme susceptibility to the virus. Primary cell culture systems, including sheep embryo kidney, rabbit kidney, dog kidney, even skin muscle and lung cells, were also successfully used to propagate fixed strains of rabies virus (Wiktor and Clark, 1975).

A concentrated and inactivated tissue culture vaccine prepared from virus grown in human diploid cell strain WI-38 has been shown to have considerably higher protective efficacy than duck embryo vaccine, as measured by the standard mouse potency test and by experimental challenge of vaccinated monkey as well as vaccinated dogs (Koprowski, 1966). Human diploid vaccine has been licensed for human antirabies treatment since June, 1980.

Since 1981 an adjuvant PHkC rabies vaccine for pre- and postexposure treatment was recommended through China (Fangtao et al., 1983). The fixed rabies virus Beijing strain was isolated from the brain of a rabid dog in Beijing, China in 1931 and had been fixed by successive passage in rabbit brains. It had been used for the manufacture of rabies vaccine for more than 30 years in China. The virus strain after being adapted to primary hamster kidney cells, is used as an inoculum in seed lots for the production of the vaccine. The virus is inactivated by the addition of formalin or phenol at certain concentrations and the type of vaccine produced is called "plain vaccine", when aluminum hydroxide is added into the plain vaccine (final concentration, 0.5 mg/ml) the product is called adjuvant vaccine. Results for pre-exposure and postexposure treatments in humans showed that the PHkC rabies vaccines are safe and effective (Fangtao et al., 1983).

A new inactivated rabies vaccine (purified chick embryo cell vaccine) has been developed using the Flury LEP-C_{25} strain of rabies virus propagated in primary chick embryo cell cultures. The antigen was purified and concentrated by continuous density gradient centrifugation and inactivated by β-propiolactone. This vaccine was tested for protective capacity in a series of laboratory tests and compared with human diploid cell (HDC)-vaccines of similar antigenicity. No significant differences have been found between the two vaccines in laboratory tests and slight advantages of one type were compensated for by advantages of the other (Barth et al., 1984).

Berlin and coworkers reported on adsorbed rhesus diploid cell rabies vaccine. The Kissling strain of rabies virus was adapted to grow in a diploid cell line derived from the lung of a fetal rhesus monkey. The vaccine consisted of virus-infected supernatant fluid inactivated by β-propiolactone and adsorbed to aluminum phosphate (Berlin et al., 1982). The vaccine is currently under medical trial. So far, apart from the Japanese chick embryo cell culture vaccine (Kondo,

1978), the Russian primary hamster tissue culture vaccine, and the human diploid vaccine, these vaccines have been used only for prophylactic vaccination in man. The introduction of a new vaccine into the area of postexposure treatment is made difficult by the fact that the use of a new, unlicensed biological product for the prevention of an invariably fatal disease should vaccine-failure occur, may create a number of medico-legal and ethical problems. However, the ethical problems can be resolved by adopting certain principles which every new kind of rabies vaccine must satisfy prior to its being applied in clinical trials for postexposure prophylaxis. Such vaccines must be provied unmistakably 1) to have superior qualities in certian important aspects when compared to licensed vaccines; 2) to provide to sensitive laboratory animals in a series of distinctive immunization and challenge schemes a degree of protection which is at least comparable to that provided by licensed vaccines; and 3) to induce in humans a serological response of a magnitude which equals or surpasses that induced by licensed products.

ANIMAL VACCINATION

Animal vaccination, together with stray dog control, is an important measure in controlling rabies in infected areas. Mass immunization of dogs is recommended in areas experiencing erzootic or epizootic canine rabies. In some countries such as the United States, the periodic vaccination of dogs against rabies is required by law in many states and localities. All vaccines must pass both potency and safety tests before being released for field use (WHO, 1966).

For dog vaccination, the World Health Organization (1966) recommended LEP (low egg passage - 40th to 60th egg passage) Flury chicken embryo vaccine in dogs 3 months of age or older. The immunity produced here is known to last at least 3 years. This vaccine is restricted to dogs but there are reported instances in which illness and death attributable to the virus vaccine following its use in cats, cattle, pet skunks and foxes. Investigations failed to reveal the presence of virus in the saliva or any reversion of the virus to virulence in such instances (Mayer, 1966).

Puppies less than 3 months old can be vaccinated safely with HEP (high egg passage - 180th to 190th) Flury strain live virus vaccine or with any of the inactivated virus preparations (nervous tissue). This vaccine, although proved safe, and may be used in any species is not very effective in cattle. Dogs vaccinated with inactivated nervous tissue vaccines should be revaccinated within 1 year. Should HEP vaccine be used in dogs the animal should be revaccinated within 2 years. One month is required for vaccines to stimulate a maximum

antibody response. Thus, dogs vaccinated during an epizootic should be confined or kept on a leash for at least 30 days after vaccination. Cats may be effectively immunized with nervous tissue vaccine of HEP Flury strain. The dosage of vaccine for cats is generally about one-half of the dose for dogs (Bisseru, 1972c).

Two types of vaccines are the most commonly used for animal rabies vaccination world-wide (Sikes et al., 1975).

1) An inactivated, phenolized vaccine of ovine origin given as a single dose inoculated intramuscularly in a dose of 3-5 ml in dogs and 1-3 ml in cats (Johnson, 1945). This vaccine is still widely used for dogs, cats, and other species of domestic animals, even though newer vaccines have been developed and used in many countries of the world.

2) Inactivated suckling mouse brain vaccine (SMBV). The SMBV is commonly used in dogs and cats, it has been used in Latin America mostly for humans since its development by Fuenzalida and Palacios (1955). It is recommended intramuscularly in dogs and cats in a dose of 1.0 ml, while in Latin America it is 2.0 ml for either dogs or cats; it is 4% suspension, inactivated by ultraviolet or β-propiolactone. This product is the most commonly used vaccine in Latin America (Pan American Health Organization, 1972). In countries where SMBV has been used, dog rabies has been controlled very successfully (Fuenzalida et al., 1965). SMBV was licensed in the United States for uses in dogs and cats since 1973.

EFFICACY OF ANTIRABIES VACCINATION

In the early days of rabies vaccine development there was no uniform standard method for evaluating the potency of a vaccine in experimental animals. Sikes et al. (1971) have shown good relation between the antigenic value of a vaccine and the effectiveness of the vaccine in producing neutralizing antibody in animals after challenge.

Vaccine Evaluation

a. Test in experimental animals

All the tests commonly employed for vaccine evaluation utilize mice. Two tests commonly used are the "Habel test" and "NIH Potency test". In the Habel test, 4 to 6 week old mice are given six intraperitoneal inoculations of 0.25 cm^2 of vaccine containing 0.5% emulsion of original wet-weight of brain over a period of 12 days. On the fourteenth day a challenge strain of virus (CVS) is titrated by intracerebral inoculation in parallel in vaccinated and control mice. To meet the minimum standards, the difference between the log of the 50% end-point in the vaccinated animals from that in the controls, should be 3 or log 1000 indicating that the vaccine affords protection against 1000 LD_{50} (Habel, 1973).

In the NIH Potency test groups of 4 week old mice are given two 0.5 ml inoculations of test vaccine intraperitoneally 1 week apart. Mice are challenged 14 days after the first vaccine inoculation with a dose of 5-50 intracerebral LD_{50} of challenging virus strain (CVS) strain virus. Two weeks after challenge the effective dose of test vaccine required to protect 50% of the mice

(ED_{50}) may be calculated. The ED_{50} of a standard NIH reference vaccine divided by the ED_{50} determined for the test vaccine gives the antigenic value (AV) of the test vaccine (Seligmann, 1973).

An antibody binding technique has been described by Arko et al. (1973) as an additional means for testing rabies vaccine. The test is based on measuring antibody combining activity of the vaccine. In this procedure vaccines are compared to a standard reference vaccine on the basis of their relative ability to absorb protective ability from a standard reference antiserum. The presence of non-adsorbed antibody is then detected in vitro by the plaque-reduction technique. The method yields results similar to those obtained with the NIH test, in a shorter time.

Recently, enzyme-linked immunosorbant assays (ELISA) were described for quantitation of rabies virus envelope glycoprotein and nucleocapsid antigens (Atanasiu et al., 1980). As rabies envelope glycoprotein is generally considered to be responsible for induction of neutralizing antibodies (Sokol et al., 1971), Van der Marel and Van Wezel (1981) recommended the use of ELISA as a fast technique for quantitation of rabies antigen especially in the standardization of inactivated rabies vaccine production.

b. Antibody response in man

The recommendations of the World Health Organization Expert Committee on rabies (1973) are mostly based upon the assessment of serum-neutralizing antibody response. Studies done by Atanasiu and his colleagues (1961, 1967) showed that the daily spacing of the inoculations appeared to be much more important than the total mass of vaccine inoculated, as SN antibody induction was delayed and antibody titers were depressed with reduced schedules of vaccination from one to seven inoculations.

Rabies Vaccination Reactions

Reactions to rabies vaccine have been recognized for almost as long as any such vaccines have been administered to man. As vaccines and their regimens for administration have been improved since those originally proposed by Pasteur, so also have the problems of prevention and treatment, however the problem of vaccine reactions still is not completely solved.

Reaction to rabies vaccine can be divided into:

1) Allergic reactions to animal tissue components of the vaccine.
2) Neurologic complications, the most serious of which is paralytic disease caused by "allergic encephalomyelitis" due to sensitivity to adult nervous tissue antigens.
3) Encephalitis caused by viable virus present in vaccines that have been insufficiently attenuated or inactivated. This besides the allergic reactions that might happen due to hyperimmune serum administered in conjunction with vaccine.

Of all the reactions to rabies vaccination neurologic reactions present by far the greatest danger. Regardless of whether vaccines contain live virus or have been inactivated by any of the various methods, all types of vaccine containing adult mammalian nervous tissue exhibit similar capacities for inducing neurologic reactions, (McKendrick, 1940). However, studies showed that the incidence of these reactions vary widely, the incidence of neurologic reactions is definitely lower among persons receiving duck embryo vaccine (Prussin and Katabi, 1964).

While the suckling mouse brain origin vaccines are also considered to be nearly free of encephalitogenic potential, their administration is not totally free of risk. Studies done by Held and Lopez' (1972) on some cases received the suckling mouse brain origin vaccine in South America, showed that the neurologic

disease seems to be clearly associated with vaccination, but they were not able to identify the component of the vaccine which is responsible for the reaction.

Although human diploid vaccine which is a tissue culture vaccine, has a greatly enhanced capacity to elicit serum neutralizing antibody formation, contain greatly reduced quantities of animal foreign protein, and provide a much better possibility of protection when compared with the nervous tissue and duck embryo vaccines, the recent report by Massachusetts Medical Society in April, 1984, showed that primary immunization with HDCV appears to sensitize some recipients to a, as yet unidentified, component of the vaccine. When booster doses of HDCV are then administered, these persons develop a hypersensitivity reaction clinically consistent with type III hypersensitivity which is presumed immune complex disease. Immunologic illness occurs 2-21 days after a dose or doses of HDCV and is characterized by a generalized pruritic rash or urticaria, the patient may also have arthralgias, arthritis, nausea, vomiting and fever.

WORLD POSITION AS FAR AS RABIES

The first organized effort to collect definitive information on the status of rabies on a global basis was made in 1959 by the issuance of the World Rabies Survey by the Veterinary Public Health Section of the World Health Organization (1959). The data collected in these annual surveys are based on replies of a questionnaire sent to each member country. The questionnaire requests data relating to the incidence of vector species, the number of human postexposure immunization to biologic prophylaxis, rabies mortality in treated and untreated individuals, and the types and quantities of rabies vaccine produced for use in man and animals.

There is always rabies somewhere in the world. According to WHO survey (1971), fifty-three countries and territories stated that they were free from rabies. A comparison with the rabies-free countries listed in 1965 showed that a number have managed to remain free; others, notably in Europe and Africa, became infected and some of these have again become rabies-free (e.g., Denmark, Gibraltar, Guam, Luxembourg, Malaysia and United Kingdom). Still others, such as Mauritania, Spain and Swaziland, are reported to have freed themselves of rabies after having remained infected for relatively long periods.

The measures adopted in these countries to maintain their rabies-free status vary from total prohibition of imports of carnivores and certain other animals from infected countries to allowing importation after vaccination and surveillance within the country. Others impose quarantine with or without precautions, including vaccination on entry with subsequent movement control or surveillance.

Animal Species Affected

Rabies virus is widely distributed in the animal kingdom. There is evidence that some animal species are more resistant to the infection than others (Lodmell, 1982). The disease exists in animals in two epidemiological forms: (a) the urban type, spread mainly by dogs, and (b) the wildlife type, mainly seen in foxes, wolves, jackals, coyotes, skunks, weasels, mongoose and bats. The virus exists mainly in four forms: classical street virus, "Oulou Fato" of dogs in Africa, paralytic bat rabies of South and Central America, and the Polar madness of Arctic foxes (Bisseru, 1972a).

According to WHO survey (1971) dogs continued to be the animals most frequently found rabid in Africa, Central and South America, Asia, and in Greece, Italy, Turkey, and Yugoslavia. Foxes in Canada and most countries in Europe. Cattle were the main species found rabid in Belgium and the Soviet Union, and were the second important species after foxes in Canada, France and Germany (Federal Republic), after dogs in Argentina, Brazil, El Salvador, Greece, Guatemala, Honduras, Iraq, Mexico, Morocco, Nepal and Yugoslavia and after mongoose in South Africa.

Skunks continued to be the principal vector species in the United States of America. Bats were implicated in South America, and North America. Species of mongoose were the main vector in Puerto Rico, South Africa and India. Jackals have become the principal species affected in Rhodesia and India.

Not only warm-blooded animals develop rabies but also domestic and wild birds may develop rabies. They may develop either the furious or paralytic form of the disease after an incubation period ranging from 6 weeks to one year. However, human rabies by the bite of a bird is rare, with a possible case of rabies in man due to infection from a cock mentioned in a book published in 1641.

Rabies in the duck was studied after discoveries of rabies strains of low pathogenicity for mammals by adaptation to chick embryo (review by Bisseru, 1972b).

Control of Rabies Among Wildlife

In the last decade wildlife species have accounted for the majority of rabies all over the world (Hayles and Dryden, 1970). Fox, skunk, raccoon, jackal, mongoose, coyote and wolf are the major problem species, but rabies has also been diagnosed in badger, bear, bobcat, deer, feral cats, muskrat, squirrel and weasel (Ballantyne and Donoghue, 1954).

A rabies epizootic among wildlife is facilitated when a particular population density among wildlife is reached or exceeded; at this level there is regular intraspecies contact that permits rapid spread of the disease by bite. However, a few researchers have suggested that epizootics are not initiated by animal to animal bite, but rather that latent virus may be activated simultaneously, in many individuals by stress (Schnurrenberger et al., 1964). Studies were done to determine the effects of population reduction in a rabies epizootic area and the spread of rabies among wildlife. Richard (1982) showed that the population reduction program along with the topography of the area and the natural barriers may have accounted for the minimal dispersals.

The method which is presently available for the control of rabies among wildlife is reduction of the vector populations to a density that will not sustain an epidemic. That reduction also results in less chance of human beings and domesticated animals being exposed.

Foxes represent a major problem in spreading rabies among wildlife in Europe. WHO studies (1981) showed that the main factors that affect the density and social structure of the fox populations are food, cover availability and hunting

pressure. Studies were done to vaccinate foxes orally in the wild with an inactivated virus in enteric-coated tablets but results showed no protection in the foxes challenged (Aubert et al., 1982) with street rabies virus strain. However, results reported by other authors, in slightly different experimental conditions and in another animal species, are not in accordance with the results in foxes (Aubert et al., 1982). According to WHO studies (1981) not only physical barriers, but also natural barriers have been identified that affect fox populations. For example:

a) area largely unsuitable as habitat for foxes, e.g. marshland.

b) urban conglomerations extended over long distances combined with lines of roads, rivers and valleys.

2) Biological barriers are more effective and permanent but depend on conditions which are not always fully understood and under control.

3) Mountains above 2000 m form formidable barriers for rabies, as do large rivers, passes, bridges and possibly tunnels form Loop-holes in these barriers.

4) Surface water and high mountains can be utilized in rabies control strategies. Buffer zones or protective belts should be considered at critical points for rabies transmission.

ANTIGENIC COMPOSITION OF RABIES VIRUS

Rabies virus is a member of the characteristically bullet-shaped rhabdovirus group. The virion consists of a helical ribonucleoprotein capsid (NC) enclosed within a lipoprotein membrane or envelope which appears to be covered with a fringe of short projections of glycoprotein in nature. Theoretically, each of these structural elements might behave as a unique antigen and each might carry one or more antigenic sites to serve for virion attachment to host cells (Sokol, 1974).

The naked viral RNA, the nucleocapsids derived from virus or from infected cells, and the viral "core particles" are not infectious when tested under conditions suitable for infection of BHK/21 cells with intact virions or with infectious RNA of other viruses (Sokol et al., 1969). The fact that naked viral RNA is also noninfectious indicates that it cannot function as a messenger RNA or be transcribed by the host-specific RNA polymerase into functional messenger RNA.

Rabies Antigen in Infected Cells

Before the development of methods for rabies virus assay, rabies infection was defined by the demonstration of rabies inclusion bodies within the infected cell. Infection of neurons examined by specific staining procedures was revealed by characteristic rabies Negri body. Fluorescent antibody staining studies by Goldwasser and Kissling (1958) gave further evidence of the rabies specific nature of these bodies. Electron microscopy of thin sections of infected cells by Matsumoto and Myomoto (1966) has shown that these cytoplasmic inclusions

consist of a moderately electron-dense matrix in which rabies virus particles may occasionally be detected.

Matsumoto (1974) reported that the bullet-shaped virus of rabies are formed by a process of budding from the cytoplasmic membrane which is closely located to the inclusion body. In susceptible tissue culture systems, virions are formed in variable quantities by budding from the cell surface. The mode of assembly seems to be quite similar to that of enveloped viruses especially of myx ovirus group.

Hammeler et al. (1968) using BHK-21 infected with rabies virus reported a different ratio of viruses within the cytoplasm to that released from the cell surface. When infected brain tissues were observed, formation of virions was completely restricted to the inside of infected nerve cells as well as glia cells. Matsumoto and Kawai (1969) based on the results of their studies suggested that the site of the rabies virus assembly varies depending on different combination of virus and host cell.

Chemical Composition of Rabies Virus

When purified rabies virions are treated with an ionic detergent desoxycholate (DOC) it solubilizes the components of the viral envelope and releases the viral nucleocapsid which can be isolated by velocity centrifugation in a sucrose density gradient (Sokol et al., 1971). Sokol (1974) reported that the nucleocapsid contains all the viral RNA and the phosphoprotein component of the virus (N), as well as the minor protein components.

The envelope fraction solubilized by treatment of the virus with DOC contains three major structural proteins, i.e. the glycoprotein (G) and the two membrane proteins (M_1 and M_2) (Sokol et al., 1971). The G protein contains three oligosaccharide side chains that account for about 11% of its total mass (Dietzschold, 1977). Cox et al. (1977) were able to solubilize rabies glycoprotein

using the non-ionic Triton X-100. This glycoprotein preparation not only reacts with and induces the production of rabies neutralizing antibodies, but also protects mice in vaccination experiments from a challenge infection with rabies virus. The glycoprotein vaccine has similar or better protective activity than vaccine made from inactivated virus (Cox et al., 1977).

Glycoprotein prepared by treatment of rabies virus with Triton X-100 and purified by isoelectric focusing was found to be homogeneous with respect to size and isoelectric point. These detergent-released materials represent homopolymers of the G protein (Deitzschold et al., 1978).

Antigens of Disrupted Virions

The disruption of purified virions with an ionic detergent (DOC), resulted in the rapid inactivation of viral infectivity and the loss of most of the hemagglutination (HA) activity; while complement-fixing (CF) activity remained unaffected (Sokol et al., 1969). Schneider et al. (1971) demonstrated that the disruption of rabies virions by natural detergent saponin resulted in the disruption and inactivation of viral infectivity, whereas rabies HA, CF, and immunogenic capacity remained largely unaffected.

The studies of the antigenic nature of the rabies virion show that it bears sites for HA, CF antigen activity, and for N antibody induction. The NC likewise, can fix complement but does not possess HA activity.

Soluble Antigens

Soluble antigens were first reported by Polson and Wessels (1953). They are naturally-occurring noninfectious antigen, complement-fixing produced by rabies virus and can be separated from the intact virion by centrifugation. Van den Ende et al. (1957) reported that SA was produced in suckling mouse brain in parallel

with infectious virus and was partially purified by precipitation at pH 4.3. They also demonstrated that the antigen they separated was stable to heating at 56°C for 30 minutes, was fairly stable at pH 6 to 10, and it maintained practically all its serological activity when treated with 0.5% phenol and 0.35% formaldehyde, but sensitive to trypsine digestion.

Kipps et al. (1957) found that the rise in infectivity preceded the first appearance of SA in mice after intracerebral injection of rabies virus and that SA continued to increase after the maximum titer of infectivity had been reached. They found that sera of immunized rabbits with SA showed the presence of both CF and N antibody, while when they are immunized with heat-inactivated SA, a CF antibody was produced by no neutralizing antibodies. Wiktor et al. (1969) concluded that SA was an immunogen for CF antibody formation as well as a vaccine which afforded protection against challenge with rabies.

INTERFERON AND RABIES VIRUS INFECTION

Although the mechanism responsible for the protection afforded by the Pasteur treatment (1882) has never been fully defined, there is evidence which indicates that factors other than the neutralizing antibody response of an individual animal play a role in preventing death from rabies (Wiktor and Koprowski, 1967; Wiktor and Clark, 1972).

Several years before the discovery of interferon, Findlay and MacCallum (1937), established the role of specific immunity due to interference between antigenically unrelated viruses.

Several studies on the growth of rabies virus in a variety of tissue culture systems showed that human diploid cells (WC-38) and rabbit endothelial cells, infected with HEP flury or CVS strains of rabies virus, were resistant to superinfection with other strains of rabies virus, as well as to a number of unrelated agents. The degree of resistance to various viruses was a function of the number of rabies virus infected cells in a culture as demonstrated by the fluorescent antibody technique. Resistance to superinfection was complete in cultures in which all cells contained rabies virus antigen. It was not possible at that time to demonstrate an interferon-like substance in fluids from these cultures (Fernandes et al., 1964; Wiktor et al., 1964).

On further studies, Crick and Brown (1974) reported that BHK-21 cells infected with LEP strain of rabies virus produce a shortened particle which contains RNA and interferes with the multiplication of the virus. When hamsters were inoculated intramuscularly with the CVS strain of rabies virus, interferon

was first detected in the brain tissue, then blood, and then simultaneously in spleen, lung, kidney and brown fat. Levels of interferon were highest in brain tissue, the most active site for virus replication, and lower in the other tissues. Thus, in this system, interferon production could be taken as an index of virus multiplication, and the appearance of interferon in the various organs demonstrated that replication of CVS rabies virus induces interferon in several tissues of the hamster (Larke, 1966).

Wiktor and Clark (1972) demonstrated the inhibition of rabies virus infection by exogenous interferon in cells of hamster and human origin. Inhibition of rabies virus by treatment of animals with various endogenous interferon inducers has also been demonstrated (Janis and Habel, 1972).

Bear and Cleary (1972) studied the role that interferon may play in inhibiting rabies infection, using animal models to evaluate the protective activity of rabies virus vaccines. The study compared the interferon-inducing capacity of duck embryo vaccine (DEV) and a concentrated, inactivated rabies vaccine [prepared from baby hamster kidney cells infected with Pitman-Moore rabies virus strain]. The results showed that interferon can be detected in the serum of the animals infected with (BHK-PM) for 3-32 hours following treatment, whereas no interferon could be demonstrated in the serum of animals innoculated with DEV. The difference in interferon-inducing capacity of the two vaccines correlated with the difference in mortality rates between the two groups of animals which received BHK-PM vaccine or DEV.

Although certain studies provide evidence that interferon may play a role in the protective activity of rabies vaccine they also indicate that other mechanisms are involved.

EARLY DEATH PHENOMENON IN ANIMALS VACCINATED WITH RABIES VACCINES

The exact way in which rabies vaccine protects against the disease is not fully understood. There are occasional failures even when vaccines of established potency have been used. In both monkeys and mice immunized with rabies vaccine and subsequently challenged with rabies virus, some die sooner than non-vaccinated controls given the same challenge (Sikes et al., 1971). This phenomenon has been termed "early death"; the mechanism underlying this phenomenon is not fully understood.

In order to study the early death phenomenon a model in mice was investigated with the results that some vaccinated mice challenged on day 2 or 3 after vaccination died earlier than animals which had received the placebo inoculation and been challenged similarly (Blancou et al., 1980). To confirm this observation, groups of 50 mice were given the challenging virus 3 days after inoculation with the vaccine or the placebo preparation. Daily cumulative percentages of mortalities in each experimental group were recorded. On day 6 after challenge, 42% of vaccinated mice wer already dead, whereas all had survived in the placebo group; on day 7, 80% of the vaccinated were already dead, while 98% of the placebo group had survived. Mortality was significantly higher in vaccinated groups than in the control group on days 8 and 9 after challenge. But by day 10 and 11, the number of animals that had died in the two groups was similar. [None of the animals in either of these two groups survived the challenge and all had died after 11 days according to the model.]

The mechanism underlying the early death phenomenon was studied by Prabhakar and Nathanson (1981). They suggested that early death from rabies virus is mediated by antibody rather than cell-mediated immune processes. It has been suggested that antibodies might enhance rabies virus infection of macrophages through their opsonization of immune complexes in a manner similar to that which has been shown to occur with other viruses (Porterfield, 1981).

King et al. (1984) studied the interaction between rabies virus strains and a particular macrophage cell line in the presence and absence of a number of different rabies virus antisera. Their results supported the hypothesis that macrophages are not only capable of supporting the replication of rabies virus but may give enhanced yields of rabies virus in the presence of rabies virus antiserum. Although their tests were carried out in vitro with a continuous line of macrophage which may differ in a number of respects from resident and elicited macrophages, it may also occur in vivo.

Thus, the interaction between virus and antiviral antibody does not always result in the neutralization of viral infectivity but it may result in consequences which are detrimental to the host.

MATERIALS AND METHODS

1. Cell Culture Medium

a. Growth medium (GM):

Growth medium consisted of Eagle's Minimum Essential Medium (EMEM) with Earl's salt (Eagle, 1959) supplemented with 10% fetal bovine serum, 10% tryptose phosphate broth, 0.1% sodium bicarbonate (10% w/v $NaHCO_3$) and antibiotics. Medium was sterilized by filtration.

b. Wash medium (PBS):

Wash medium consisted of calcium and magnesium-free phosphate buffer saline, pH range 7.48-7.5. The medium is sterilized by autoclaving for 15 minutes at 15 Lbs PS and 121°C.

c. Resuspending medium (RM):

Resuspending medium consisted of EMEM, without serum supplemented with 0.1% sodium bicarbonate medium was sterilized by filtration.

d. Freezing medium:

Freezing medium consisted of EMEM with, 10% fetal calf serum, 10% dimethylsulfoxide, and 0.05% sodium bicarbonate adjusted pH to 7.0.

2. Rabies Virus - MDPH

The virus strain used for the vaccine preparation was the Kissling strain, provided by Michigan State Department of Public Health. Originally, the virus strain was obtained by Michigan State Department Public Health at passage 112. The virus was passed four times in Baby hamster kidney cell culture (BHKS) and

thirteen times in a diploid cell line developed from the lung of a fetal rhesus monkey (FRhL-2). This virus was used to innoculate the primary goat kidney cells.

Challenging virus:

The challenge virus used in the NIH mouse potency assays was a fixed rabies virus strain, CVS 11. Provided by Michigan State Department of Public Health (MDPH).

The challenge virus for post-exposure experiments was rabies street virus Lot 186 prepared at the MDPH from fox salivary glands and stored at -78°C.

3. Animals:

a. Guinea pigs were English, short-hair, albino stock designation Mdh:(SR(A)), obtained from the Michigan Department of Public Health Laboratory Animal Breeding Facility.

b. Mice were Swiss-Webster albino stock designation Mdh:(CFW), from the MDPH Laboratory Animal Breeding Facility.

4. Primary Tissue Culture Preparation:

A one-day-old male, grade Nubian goat was obtained from a goat farm in rural Mason, Michigan, was used for this study.

a. Kidney tissue preparation: Goat kidneys were removed from the animal with aseptic surgical technique and immediately transferred to a sterile container. Kidneys were placed in a petri dish. The entire kidney was minced into fragments from 1-3 mm in size. The minced tissue was transferred to a 125 Erlen-Meyer flask containing a magnetic stirring bar.

b. Trypsinization: The tissue mass was covered with 0.25% trypsin, pre-warmed to 37°C. The tissues were stirred for 10-12 minutes. The tissue mass was allowed to settle, and the supernatant fluids were decanted into a cold

receptacle containing 1 ml of bovine serum. Fresh trypsin was added to the trypsinizing flask and the above procedure repeated until amount of remaining tissue was negligible (4-6 times). The collected cell suspension was centrifuged for 10 minutes at 500 g. The supernatant was removed, cells were washed with PBS, and resuspended in GM and filtered through two layers of cheesecloth to remove large clumps. Cells were counted, using a hemocytometer to determine the number of cells in the suspension. Only cells showing nuclei and attached cytoplasm were counted. Clumps of cells were enumerated as a single cell. Plastic tissue culture flasks (150 cm^2) were prepared with 30-40 ml of GM. Flasks were innoculated with $10x10^6$ to $20x10^6$ of cells, and incubated at 37°C for 24 hrs. The GM was removed and flasks were washed with PBS (10-15 ml), then fresh GM was added. After 3 to 4 days incubation at 37°C a confluent cell sheet was obtained. The GM was discarded, flasks were washed with PBS and the cell sheet was covered (3 to 4 ml) with 0.25% trypsin at 37°C with the cell side down until cells appear to flow off the flask (3 to 5 minutes). Trypsinized cells from several flasks were resuspended in GM, centrifuged, washed and resuspended in known volume of GM. Cells were counted, and used to reinoculate flasks at a concentration of $7x10^6$ to $10x10^6$ per flask. The same procedures were followed for incubation and trypsinization of the cells.

c. Freezing of goat kidney cells: Cells, after being trypsinized and washed, were counted, resuspended in a freezing medium at a concentration of $20x10^6$ per ml and distributed in 1 ml amounts into ampules. Ampules were then sealed using a "propane torch" and stored immediately at -60°C and finally stored in liquid nitrogen.

When cells were needed vials were rapidly thawed in a 37°C water bath and the cells removed and diluted with GM, washed, tested for viability and used for inculating new flasks. Cells used for all experiments were grown under the

same conditions, using the same formulation of the growth medium. Cells were allowed to grow to pass four and used for rabies virus inoculation. Cells were plated at a concentration of $7x10^6$ to $10x10^6$ per flask.

Potency Test:

Potencies of vaccine preparations were determined by the NIH Mouse Potency Test (Seligman, 1973). The test is based on the use of white Swiss-Webster albino mice, approximately 4 weeks old, uniform in weight (11-15 gm) and of one sex. The challenge virus strain, CVS 11, was supplied by MDPH.

a. Immunization of mice: Three or more dilutions of each vaccine under test are prepared in buffered saline using five-fold increments. At least 10 mice are injected intraperitoneally with 0.5 ml of each dilution vaccine. Two doses of vaccine are given to each mouse one week apart. Enough control mice are set aside at the time the test mice receive the first dose of vaccine, so that an adequate titration of the challenge virus can be made with at least 10 mice on each dilution of virus.

b. Challenge of the control and test mice: All mice are challenged intracerebrally 14 days after the first dose of vaccine. The control mice (10 mice for each dilution) are divided into groups and given the same dose (0.03 ml) intracerebrally of at least four ten-fold dilutions of the challenge virus in order to determine the LD_{50}. The group of control mice is always inoculated with the test dose of virus after all immunized mice have been inoculated. The potency of the challenge virus for each potency test should have a calculated LD_{50} value of between 5-50 LD_{50}. All mice are observed for 14 days from the time of the challenge injection. Those deaths occurring after the fifth day and preceded by signs of rabies (paralysis, convulsions) are considered rabies deaths. Any mice

becoming paralyzed, but surviving the 14-day observation period, are considered the same as deaths due to rabies (Seligman, 1973).

The definitions of "paralysis" and "convulsions" as applied to mice following the injection of the challenge dose are as follows:

Paralysis is the partial or complete loss of motor power to one or more legs.

Convulsions are indicated by the violent and abnormal muscular contraction of the body, often termed spasms. These are brought about by external stimulation, such as disturbing sound or handling.

c. Calculation of potency: Fifty percent endpoints are determined for both the reference and test vaccine by the method of Spearman and Karber (Finney, 1952):

$$\text{Log}_{10} \text{ endpoint dilution} = - x_o - \frac{d}{2} + d\frac{r_i}{n_i}$$

x_o = $\log_{10}$ of the reciprocal of the lowest dilution at which all animals are positive.

d = $\log_{10}$ of the dilution factor.

n_i = number of animals used at each individual dilution (after discounting accidental death).

r_i = number of positive animals (out of n_i). Summation is started at dilution x_o.

ED_{50} of the vaccine titration is the dilution that protects, half of the animals.

LD_{50} of the virus is the dilution that kills half of the animals.

d. Rabies Virus Titration:

Ten-fold serial dilutions of the virus were made. Mice were divided into groups of ten each and inoculated intracerebrally with 0.03 ml of proper virus dilution. Mice were observed for 14 days from the day of injection. All animals dying before the fifth day, were considered dead for reasons other than rabies. The Spearman and Karber (Finney, 1952) method was used to calculate the virus

LD_{50}. In case of virus titration the number of positive animals are the number of dead animals due to rabies.

Neutralizing Antibody Determination:

Serums from pre-exposure vaccination and post-exposure vaccination experiments were tested for neutralizing antibody by the Rapid Focus Fluorescent Inhibition Test (Smith et al., 1973). In this test CVS rabies virus fixed strain is mixed with serial dilutions of test serum, the virus-serum mixture was added to a BHK cells and incubated for 24 hours. After the incubation period the cell monolayers were fixed and examined for the presence of non-neutralized rabies virus by fluorescent antibody staining.

a. Medium: EMEM with 10% fetal calf serum, 10% tryptose phosphate broth. This medium was used for diluting the virus and for the growth of the baby hamster kidney cells (BHK) which is needed for the test. Baby hamster kidney cells were maintained at MDPH Laboratories.

Preparation of the serum: Serum was diluted 1 to 5 (0.1 ml serum + 0.4 ml medium); 2) serum dilutions was heat inactivated at 56°C for 30 minutes; 3) five-fold dilutions of serum were prepared to get dilutions 1 to 25, 1 to 125 and 1 to 625.

b. Neutralization: Rabies virus for challenging was diluted (1/100). 0.4 ml of 1/100 virus dilution were added to each of the tubes (except to back titration). Tubes were mixed and incubated at 37°C for 90 minutes in a CO_2 incubator. The incubated serum-virus mixture was pipetted into culture microplates (0.2 ml/well). BHK cells ($1x10^6$/m) were added to each well in equal volumes (0.2 ml/well).

c. Back titration of the challenging virus: After incubation at 37°C for 90 minutes, virus was diluted [0.4 ml of the first tube transferred to the second tube

which contains 0.9 ml of medium] up to the fourth tube. 0.2 ml of each virus dilution was transferred to the microplate; 0.2 ml of BHK cells were a[illegible]

Microplates were incubated at 37°C for 24 hours in a CO_2 incubator.

d. Fixation: Plates were removed from incubator, and supernate discarded. Washed with PBS twice, plates were fixed with alcohol 70% for 15 minutes. Alcohol was discarded, plates were washed with PBS and distilled water.

Staining: Antirabies fluorescent conjugate was diluted and added to each well. Plates were incubated at 37°C for 30 minutes. Plates were washed with PBS and distilled water, air dried.

e. Detection of non-neutralized rabies virus: Plates were examined with a fluorescent microscope. Twenty low-power (160X) microscope fields were observed for each dilution and the number of fields containing fluorescing cells were tabulated.

Rabies Virus Propagation:

Primary goat kidney cells at passage level four were used to grow the rabies virus. Cells were suspended in GM, inoculated with rabies virus at the required ratio. Flasks were incubated at 37°C, and samples collected according to the set schedule. Control cells were set aside to detect any change in the cell growth during the incubation period. The rabies virus was titrated as described.

Rabies Virus Inactivation and Vaccine Preparation:

Primary goat kidney cells were inoculated with enough virus to give one virus particle for each 10-20 cells. Virus was harvested at the end of the incubation period (13-15 days) and was centrifuged, filtered and inactivated. The inactivation was performed by adding sufficient beta-propiolactone to obtain a final concentration of 0.025% and stirring the mixture at 4°C for 24 hours followed by 2 hours at 37°C. Samples were taken to test for virus inactivation.

The inactivated virus was concentrated by the addition of 0.15 mg of aluminum phosphate per ml of fluid. The mixture was stirred for four hours and allowed to stand overnight at 4°C to insure complete adsorption. The precipitate was washed with WM and resuspended in 1/20 of the original volume in RM.

Experimental Design:

a. Antibody induction: Batches of GkRV adsorbed on aluminum phosphate were tested on guinea pigs for antibody induction. Animals were given 0.1 ml of the vaccine (the tested or reference vaccines) intramuscularly on days 1 and 14, or on days 1, 7 and 14. All animals were bled 14 days after the last vaccination.

Antibody titers were determined using the RFFIT technique as described.

b. Post-exposure experiments: Simulated post-exposure in guinea pigs was done using two batches of GkRV. Groups of animals (10 each) were challenged in the footpad with 0.03 ml of $10^{-3.5}$ ED_{50} of street rabies virus Lot 186. Animals received vaccinations on days 1, 3, 7 and 14 as intramuscular injections. Control animals were divided into four groups, each group was inoculated in the footpad with a dose of 0.03 ml of several 10-fold dilutions of the virus.

RESULTS

Trypsinized cells were grown to a confluent monolayer in plastic flasks or in glass bottles. Eagle's Minimum Essential Medium with Earl's salt supplemented with 10% fetal bovine serum was chosen because of economics and availability. After the formation of monolayers (3-4 days), cells were washed, trypsinized, counted, and plated in more flasks at a concentration of $7x10^6$ - $10x10^6$ per flask. Cells were passaged until passage five without any noticeable change in the morphology. The cells had the characteristics of normal cells in culture. Cells adhered firmly to the flask surface about 2 hours after subcultivation, and they formed a full sheet within 3-4 days. Cells at subculture two, three and four were kept in freezing media and stored in liquid nitrogen. It is apparent that subculturing cells, can result in one to get a full sheet of cells, and when those cells are infected with rabies virus it was much easier to identify any cytopathic effect under the microscope, than if the cells were infected at passage zero.

Goat kidney cells suspended in GM were infected with MDPH rabies virus fixed strain, at three different multiplicities -- 1:10-1:20 and 1:100 of virus particles to cells. Samples were collected on days 2, 5, 7, 9, 12 and 17. Virus titrations were estimated by the mouse inoculation test (Table 1). Virus titration indicated that the virus reached a peak in about 5 days and then started to drop (Figure 1). By comparing the three multiplicities of the virus it can be seen that eventhough the 1:100 multiplicity output was low in the first 2-3 days it reached the peak during the same period of time as the other multiplicities, and that all of the 3 multiplicities are comparable. Results in Table 2 show that rabies virus was

Table 1

Rabies Virus Growth in Primary Goat Kidney Cells at 3 Different Multiplicities

Day Sample Taken	Log_{10} mLD_{50}/ml[1]		
	1:10[2]	1:20[2]	1:100[2]
2	5.1	4.9	2.5
5	8.0	8.0	8.0
7	7.6	7.4	6.8
9	7.2	6.6	6.7
12	6.2	6.0	6.3
17	5.8	6.1	4.8

[1] Log_{10} mLD_{50} were calculated using Spearman and Kerper equation. Samples were collected on days 2, 5, 7, 9, 12 and 17.

[2] PGKC were inculated at 3 different M'I - 1:10, 1:20 and 1:00 of MDPH rabies virus to cells.

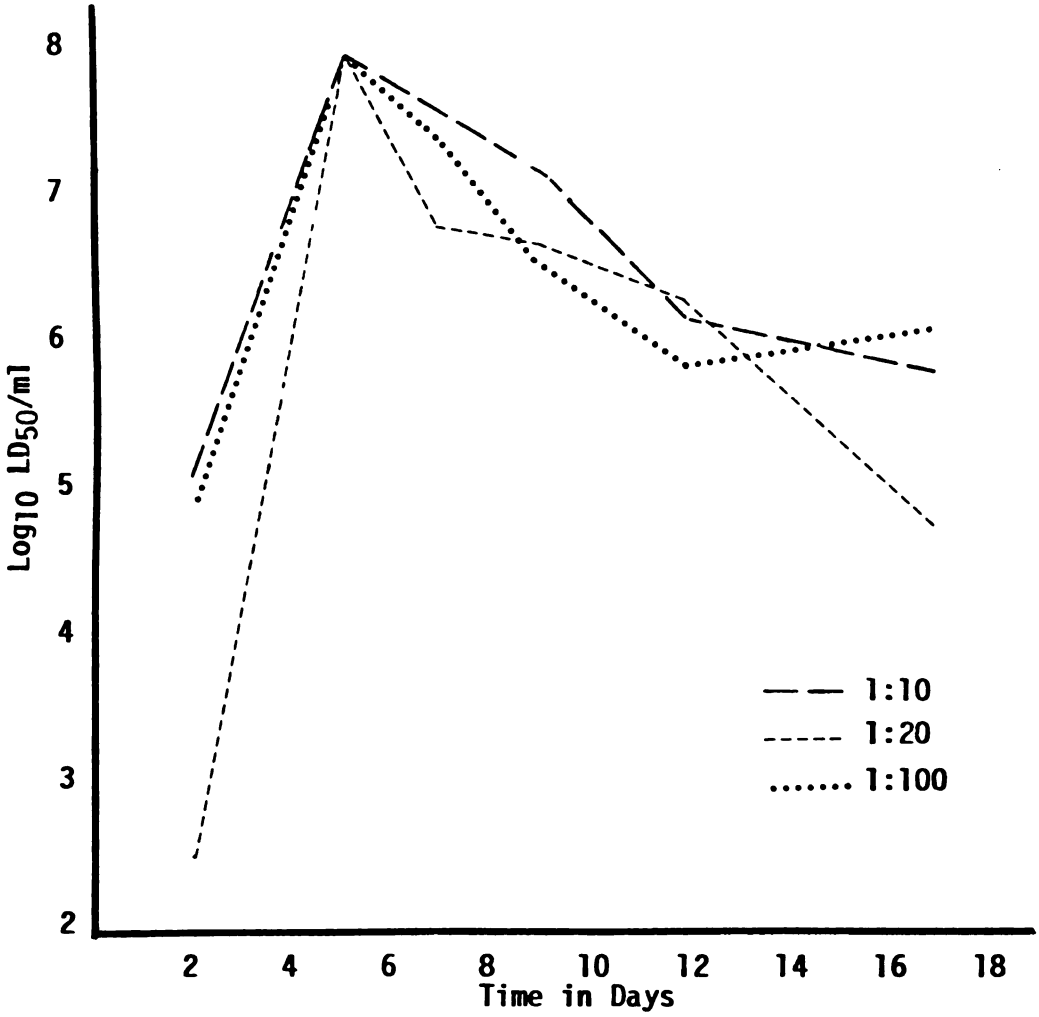

FIGURE 1. Rabies virus growth curve at 3 different multiplicities.

1) Goat kidney cells were inoculated with MDPH rabies virus fixed strain at 3 different multipilicities.

2) Samples were collected on days 2, 5, 7, 9, 12, and 17. Rabies virus were titrated on mice.

3) ED_{50} = reciprocal of dilution of virus that kills 50% of the animals.

Table 2

Titer of MDPH Rabies Virus from PGKC
at Different Passages

Virus Passage Level[1]	Media Used	Day of Sample Taken	Virus Titer[2]
1	GM	19	4.5
2	RM	14	6.4
3	RM	14	6.3
4	RM	14	5.9

[1]Passages 2, 3 and 4, cells were inculated as monolayers and incubated at 33°C. Passage 1, cells were in suspension and incubated at 37°C.

[2]Virus titer determined in mice and expressed as the log LD_{50}/1 ml.

able to keep its infectivity when it is passed in primary goat kidney up to passage four.

Figure 2 shows the basic steps in vaccine production. Cells were allowed to grow to form monolayers, washed and inoculated with the virus and allowed to incubate in RM (serum free) for 13-15 days. Virus was harvested, inactivated with BPL at a concentration of 0.025%, and samples were taken to test for virus inactivation. Rabies virus antigen was concentrated by adsorption to aluminum phosphate to give the final product. Potencies of the adsorbed rabies virus vaccine which were determined by the NIH Potency Test. Table 3 results show that the ED_{50} of the goat kidney rabies vaccine were comparable to the NIH reference vaccine and the HDCS vaccine.

Table 4 shows results of antibody induction in guinea pigs. Animals were vaccinated with goat kidney rabies vaccines, NIH reference vaccine on days 1 and 14 and the antibody levels were determined on day 28. Figure 3 shows the relation between the antibody response of guinea pigs to vaccine dilutions calculated on day 28 and the antigen content of each given dose. Results for antibody induction on guinea pigs given injections on days 1, 7 and 21 and bled 14 days later is shown in Table 5, goat kidney rabies vaccine I and human diploid vaccine were used for this experiment.

Results in Table 4 show that guinea pigs responded well to the goat kidney rabies vaccine they received. Although V_2 vaccine is of a lower potency than the reference vaccine and the other two goat kidney vaccines, the antibody response of the animals receiving V_2 is still comparable. Results in Table 5 show that the number of animals responded at higher dilution of the goat kidney vaccine are more than the number of animals responded in case of human diploid vaccine used under the same schedule and dilution.

INOCULATION OF CELLS

Multiplicity of 1:10 virus/cell
13-15 days incubation at 33-35°C

HARVESTING OF VIRUS

Clarification by centrifugation

INACTIVATION OF VIRUS

Beta-Propiolactone
Final concentration, 0.025%

CONCENTRATION OF VIRUS

Addition of 0.15 mg/M of
aluminum phosphate

FINAL VACCINE

20X concentrated

Figure 2. Production of rabies vaccine in primary goat kidney cells.

Table 3

Potencies of Trial Vaccines Produced by the Method Described in Figure 2

Vaccine Tested	Potency I.U.	Antigenic Value	Challenge Virus LD_{50}	Ratio to Standard[1]
$GKRV_1$	2.3	0.38	15.0	34.6/91.2
$GKRV_{4_B}$	4.3	0.72	79.4	29.5/40.7
$GKRV_{4_C}$	7.0	1.1	79.4	40.7/34.6
$GKRV_6$	5.5	1.4	79.0	17.4/13.2
$GKRV_7$	4.0	1.0	79.0	13.2/13.2
$GKRV_8$	9.5	2.4	79.0	31.6/13.2
$GKRV_I$	27.6	6.9	79.0	91.2/13.2
$RDRV_{104}$[2]	4.3	0.73	79.4	25.1/34.6
RCC_3[3]	6.0	1.0	79.4	34.6/34.6
HDCV[4]	8.6	1.45	79.4	50.0/34.6

Seven lots were tested for potency by the mouse potency test (NIH), in comparison with reference vaccines.

[1] ED_{50} of tested vaccine/ED_{50} of reference vaccine.

[2] $RDRV_{104}$: Rhesus diploid rabies vaccine lot 104 (MDPH).

[3] RCC_3: National Institute of health (NIH) reference vaccine.

[4] HDCV: Human diploid cell vaccine.

Table 4

Antibody Response of Guinea Pigs to Decreasing Doses of Rabies Vaccine[1]

Vaccine Tested	Potency I.U.	Dose[2]	Route	Antibody Response	
				NO of Animals Responded	Mean Serum I.U.
V_2	1.6	0.1	I.M.	6/6	29.0
		0.01		6/6	27.9
		0.001		6/6	< .1
V_{4B}	4.3	0.1	I.M.	6/6	31.3
		0.01		6/6	14.3
		0.001		6/6	1.0
V_7	4.0	0.1	I.M.	6/6	24.3
		0.01		6/6	14.6
		0.001		6/6	< 1
RCC_3[3]	6.0	0.1	I.M.	6/6	39.3
		0.01		6/6	28.5
		0.001		6/6	5.5

[1]Guinea pigs were vaccinated on days 1 and 14, and bled 14 days later.

[2]Animals received an injection of 0.1 ml of the exact dilution given intramuscularly.

[3]RCC_3 is an NIH reference vaccine (MDPH).

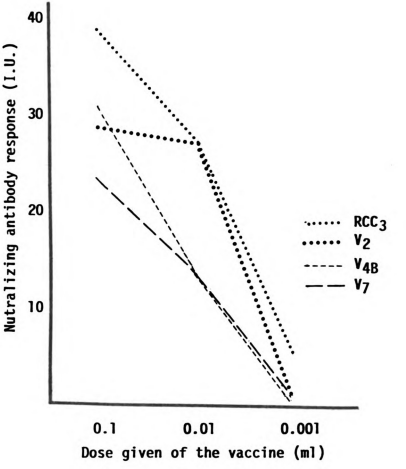

FIGURE 3. The relation between the antibody response of guinea pigs and decreasing doses of trial vaccine given I.M.

1) RCC_3: NIH reference vaccine. V_2, V_{4B}, and V_7: goat kidney rabies vaccines.

2) Animals were vaccinated on days 1 and 14 and bled 14 days later.

3) Each point represents the mean titer for all animals in each group.

Table 5

Antibody Response of Guinea Pigs to Decreasing Doses of Rabies Vaccine[1]

Vaccine Tested	Potency I.U.	Dose[2]	Route	Antibody Response	
				NO of Animals Responded	Mean Serum I.U.[3]
Vaccine I	2.3	0.1	I.M.	6/6	29.1
		0.001	I.M.	5/6	4.4
HDCV[4]	8.6	0.1	I.M.	6/6	26.3
		0.001	I.M.	2/6	< 1

[1]Guinea pigs were vaccinated on days 1, 7, 21 and bled 14 days later.

[2]Each animal received 3 doses of 0.1 ml of the dilution intramuscularly as indicated in the table.

[3]Serum neutralizing antibody (I.U.) tested by the REFIT test. I.U.s are calculated as the arithmatic mean titer for all animals in each group.

[4]HDCV: Human diploid cell vaccine.

Results from post-exposure vaccination experiments are shown in Table 6. Goat kidney rabies vaccine numbers 1 and 8 were used to vaccinate guinea pigs on days 1, 3, 7 and 14. Animals were challenged on day zero with MDPH street rabies virus lot 164 at a dose of 47 G.P LD_{50}/0.03 ml in the footpad. As indicated in Table 6 all animals received doses of undilute vaccine I were completely protected from rabies, while 5 out of 6 guinea pigs died (83% mortality) in the non-vaccinated control group. One guinea pig died out of the group received vaccine 8 (undilute). All surviving animals had good antibody titer at day 60.

Table 6

Results of Post-exposure Vaccination Experiment in Guinea Pigs

Treatment (Trial Vaccine)	Vaccine Dose[1]	Challenge Virus Dose[2]	Mortality[3]	Medium Days of Death	Antibody Response (I.U.)[4]
Vaccine I GKRV	0.1	47	0/10	0	16.4
	0.01	47	0/10	0	18.0
	0.001	47	2/10	19 days	16.7
Vaccine 8 GKRV	0.1	47	1/10	23 days	8.2
	0.01	47	1/10	21 days	9.0
	0.001	47	5/10	19 days	6.1
None (controls)[5]	---	470	6/6	20 days	0
	---	47	5/6	26 days	9.4
	---	4.7	5/6	40 days	22.0
	---	0.47	3/6	41 days	> 1

[1] All animals were vaccinated on days 1, 3, 7 and 14 intramuscularly with a dose of 0.1 ml of the exact dilution as indicated in the table.

[2] All animals challenged on day zero with street rabies virus strain Lot 146 (MDPH) at a dose of 47 $GPLD_{50}$/0.03 ml given in the footpad.

[3] Mortality: is the number of animals positive for rabies to the total number of animals at risk in each group.

[4] Serum neutralizing antibody was determined by REFIT test at day 60.

[5] Control animals were divided into four groups. Each group received a dose of 0.03 ml of ten-fold dilution of the street virus in the footpad on day zero.

DISCUSSION

Specific prevention of rabies in man through the use of vaccine represents an old scientific aspect of rabies treatment. Most efforts that have been made in recent years attempted to develop a practical vaccine from some virus source other than infected adult mammalian brain tissue in order to reduce or eliminate post-vaccinal complications involving the central nervous system.

The establishment of a tissue culture source of virus for vaccine production has always been hard to accomplish for the following reasons: 1) Virus yields in most tissue culture systems are too low for making a potent inactivated virus vaccine; 2) the cells used in tissue culture vaccine must be normal, free of extraneous infectious agents and readily available in large quantities; 3) the tissue culture medium should be free of any possible sensitive foreign antigens such as heterologous serum. A variety of primary cultures from adult tissue, primary culture from embryonic tissue, and cultures of continuously cultivated cell lines have been used as targets of rabies virus infection. The fear behind the use of continuous cell lines in the production of vaccines for human use is due to the possibility that virus vaccines, with intact virions might have cellular nucleic acid incorporated within the virions themselves, and so, if the continuous cell line DNA has transforming potential, the vaccine virus might transmit such sequences to the vaccine recipient.

However, recent studies with human diploid cell strain rabies vaccine, proven to be protective for post-exposure and pre-exposure use for humans, it showed that a hypersensitivity reaction occurs especially when persons receive a

booster dose of the vaccine (Morbidity and Mortality weekly report, April, 1984). The use of primary cells has been recommended as a safe substrate for vaccine production especially if the virus is completely inactive (Petricciani et al., 1981). The aim of this study was to investigate the use of kidney cells, taken from a one-day-old baby goat, as a substrate for propagation of rabies virus to be used for vaccine production for human use.

According to Hayflick and Moorhead (1961), definitions of "cell line" which apply only to those cells that have been grown in vitro for extended periods of time (years) will be inapplicable to the goat kidney cells. They have chosen to refer to the human diploid cells as "cell strain" which is defined as a population of cells derived from animal tissue subcultured more than once in vitro, and lacking the property of indefinite serial passage while preserving the chromosomal karyotype characterizing the tissue of origin. The term "primary cells" should indicate those cells obtained from the original tissue that have been cultivated in vitro for the first time. If subsequent passages of these cells are made, it is assumed that such cells can properly be called a cell strain until they are either lost through further subcultivations or alter to a heteroploid state, in which case they could properly be referred to as a cell strain. Knowing that human diploid cells can be passed more than 30 times, and knowing that goat kidney cells can be used at passage one or two for vaccine production, I considered them primary cells. Subculturing cells not only allows one to get a full sheet of cells, but also it provides a very large number of cells which can be stored, and allows cells to be available at any given time in numbers large enough for experiments. Cells restored from the frozen state after 31 months still retained all the characteristics of normal cells and good viability. When goat kidney cells were allowed to grow and form a full sheet cells stopped dividing, proving the concept of contact inhibition between normal cells (Abercrombie and Heaysman, 1954).

A number of factors, including the type of cell culture, the strain of virus and input multiplicity of infection, the incubation temperature, the pH of the medium, and the protein supplement of the medium have been found to influence the quantity of rabies virus obtained (Clark and Wiktor, 1972). Maximal rabies virus titers are obtained in cultures; incubated at a temperature of 32-33°C, when the culture medium supplemented with protein and the pH toward the alkaline side especially at the initiation of virus infection. Virus titer alone cannot provide the basis for judgment as to the optimal properties of the culture yielding virus for vaccine preparation. "Late" cultures with diminished virus titers may still contain considerable amounts of antigen. So the antigenic value of the virus preparation is what is important for vaccine production, not the virus titer (Perkins, 1974).

Results on guinea pigs for antibody induction experiments indicated that antibody responses for animals vaccinated with goat kidney rabies vaccine were comparable to those from the NIH reference vaccine and HDCS vaccine given under the same sched··le. Observations of the animals showed that GKRV was well tolerated; no local reactions were detectable nor were severe side effects. The antibody induction study with two injections of each vaccine on days 1 and 14 revealed that the total antibody induction of all vaccine dilutions calculated on day 28 is proportional to the antigen content of each given dose. Higher vaccine dilutions induced higher antibody levels.

In the post-exposure vaccination experiments, guinea pigs were given the vaccine (GKRV) only; no rabies antiserum was given. A significant protection was achieved when they were challenged in the footpad with a dose of 47 $GPLD_{50}$ of street rabies virus. Results with $Vaccine_8$ are difficult to interpret because one animal died out of the group that received undilute vaccine. This was more likely due to what has been called early death phenomena than due to inadequate

vaccination, and this was supported by the number of animals which survived in the group that received 10^{-1} of the same vaccine (9/10). This phenomenon is not clearly understood.

However, results on guinea pigs for antibody induction and post-exposure treatments showed that goat kidney rabies vaccine induces a reasonable antibody titer and were able to protect animals when they were challenged with street virus, still some questions need to be answered: for example, how soon an antibody would be detected in the serum after immunization, how long it will stay high in the blood, when a booster dose is needed, and how many doses of the vaccine to be given to insure complete protection, etc. To answer these questions, more experiments should be done with animals to prove that individual variations in animal response may occur but overall results will not alter the value of using goat kidney cells for rabies vaccine production.

CONCLUSIONS

The greatest value of antirabies vaccine or treatment in humans is in the protection of patients who are actually infected by rabies virus. Since the human diploid cell strain rabies vaccine is too expensive to be used, especially in the developing countries, this study was done to investigate goat kidney cells as a substrate for a tissue culture rabies vaccine. However, the work which has been done on goat kidney rabies vaccine is only a portion of what needs to be done (testing for stability, sterility tests, safety, post-exposure and pre-exposure experiments on animals) with biologic produts before they go into clinical trials on humans. Results obtained in this study clearly indicate that it is possible to prepare a safe rabies vaccine from primary goat kidney cells in a reasonable time and at a reasonable price. Experiments showed that rabies virus will grow in goat kidney cells, without the need for adaptation. Primary cells in the first few passages, are easy to work with, they can grow on microcarier which would be helpful for mass production. Inactivation of the harvested virus before concentration makes handling of the virus less hazardous. Adsorbing the virus on aluminum phosphate has the advantage that not only does aluminum phosphate act as an adsorbing agent for concentrating the vaccine but also it acts as an adjuvant which helps to develop high antibody titer which are sustained for a longer periods of time.

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