CHAPTER 1

INTRODUCTION

1.1 CHARACTERISTIC OF RABIES VIRUS

Rabies is an infectious diesease characterized by an acute and profound dysfunction of the central nervous system. It is caused by a virus which has a natural habitat in the salivary gland. All warm blooded animals are susceptible to the virus which is transmitted generally through bites (12). There are two types of rabies : the natural disease as it occurs in enzootic form in wild life (e.g. wolves, mongooses, foxes, coyotes and bats), and the encephalitic or urban type, which affects domestic animals (e.g. dogs, cats, and cattle) and occurs primarily in dogs (12). Dogs are also the main source of infection in man. Rabies in dogs is clinically classified as either "dumb" or "furious". In the dumb type, the paralytic signs develop early, and in the furious type the excitation phase is prolonged. In most cases, infected dogs present same manifestations of both types, mainly an excitation phase with irritability, restlessness, and viciousness followed by depression and paralysis. Rabies in cats is generally characterized by excitation, viciousness, and erratic behavior. Cattle also usually have the furious type of rabies. The animals became aggressive and move about violently, but they rarely bite. They generally die after a period of prostration and complete paralysis of the muscles of locomotion. Rabies in horses, mules,

donkeys, sheep, and swine also is marked by excitation, and resembles the disease in cattle (12).

Experimental infection can be established in hamsters, mice, guinea pigs, white rats, and rabbits, in order of decreasing susceptibility. Animals, such as wolves, mangooses, bats, foxes, skunks, and so forth, are also used for experimental studies with rabies virus (12).

The modern aspect of research on this disease was initiated by Pasteur and his associates, who altered the viral pathogenicity by serial intracerebral passage in rabbits. This resulted in the development of variety of rabies virus which could be used for vaccination. The modified virus, with a short incubation period, was called "fixed" in order to distinguish it from the "street" virus found in nature (12). Fixed virus used to produce vaccines, or employed in diagnostic and research procedures, have low pathogenic properties when inoculated peripherally in low doses. This included all such strains as CVS*, LEP**, and HEP***. Street virus especially in the presence of hyaluronidase containing saliva, ability to infect and cause disease in animals is mainly a function of virus dosage. The susceptibility of man to small amounts of street rabies virus is apparently not as great as that of foxes and cattle, but since human infection has been known to occur even after relatively small puncture wounds, on the

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* CVS = Challenge Virus Standard

** LEP = Low Egg Passage

*** HEP = High Egg Passage

fingers for example, it is wise to consider all wounds with street virus as potentially very dangerous (17).

The discovery by Negri of characteristic intracytoplasmic inclusion bodies in neurons of animals and human beings infected with rabies made possible a prompt microscopic diagnosis of this disease. Hoyt and Jungeblut used white mice for rabies diagnosis because street virus innoculated into these animals has a short incubation period and always produces Negri bodies (12).

Nature of virus particles

The morphology of the rabies virus particle has been further studies in cell cultures and tissues from infected animals. Rabies virus has been classified on a morphological basis as a rhabdo virus, a group that includes many viruses from mammals, reptiles, fish, insects, and plants (37).

Rabies virus RNA is single-stranded, with a molecular weight of 4.6×10^6 amu* and a sedimentation coefficient of 45S**. By electron microscopy, the virus particles (virions) localized by ferritin-tagged specific gamma-globulins the virions appear as a rod-like particles usually with one round and one flat end. This gives them a "bullet-like" shape with a fairly constant diameter of

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* amu = Atomic mass unit

** S = Sedimentation coefficient

75-80 nm* and a length of about 180 nm. The length may vary under different conditions of replication (15, 34). In tissue cultures, at least, the longer forms seem to be associated with street-virus strains and the short forms with the fixed strains of rivus (33). The virion is composed of a helical nucleocapsid surrounded by a membrane bearing surface spikes that have a knob-like structure at the distal end. The membrane does not completely cover the flat end of the virion. By a negative staining technique, it is occasionally possible to reveal distinct surface arrangement of hexagons forming a honey comb. The nucleocapsid is a single stranded right-handed helix (37). On material partly purified by column adsorption or by centrifugation the same morphological aspects are observed. Studies on the multiplication of rabies virus in tissue culture show that rabies antigen appears in the perinuclear cytoplasm approximately 15 hours after infection of the cell. Later, the virus appears throughout the cytoplasm and virus particles can be seen separating from the cell wall. At the time when tissue culture fluids contain the maximum amount of virus, the cytoplasm of infected cells shows partial autolysis with severe metabolic disturbances and some cells appear to be almost filled with virus particles (36).

Rabies virus contains at least 4 major protein components detectable by electrophoretic fractionation. The component with the

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* nm = nanometer

largest molecular weight (80,000 amu) is a glycoprotein that constitutes the protein moiety of the spikes protruding from the viral envelope. The second largest polypeptide (M.W. 62,000 amu) corresponds to the protein of the viral nucleocapsid. The protein moiety of the nucleocapsid isolated from infected cells is identical with that of the virion. The remaining protein components (M.W. 40,000 amu and 25,000 amu) are constituents of the virus membrane (37).

Rabies virus is rapidly inactivated by lipid solvents and 0.1% trypsin. The virus is relatively stable at pH 5-10 at 4°C, but inactivated rapidly at pH 3 or 11. Virus suspended in 0.1% bovine serum albumin at near neutral pH is inactivated, with a half-life of about 4 hours at 40°C and about 35 seconds at 60°C, but it is stable for several days at 0-4°C and for several years when frozen at -70°C or freeze-dried and held at 0-4°C (37).

Structural antigens

Rabies virus particles contain two distinct, major antigens; a glycoprotein antigen from the virus membrane and an internal nucleoprotein antigen. The glycoprotein seems to be the only antigen capable of inducing the formation of virus neutralizing antibodies and protecting animals against subsequent challenge with rabies virus. The nucleoprotein antigen induces the formation of antibodies demonstrable by the complement fixation and precipitation techniques. Animals immunized with nucleoprotein antigen are not protected against challenge infection. Strains of virus isolated from naturally infected

animals from different parts of the world may vary as regards pathogenicity for experimental animals, but are very similar when studied by the cross-protection test (37).

Dynamics of virus replication in vitro

(1) Optimal conditions for in vitro growth of infections virus

A number of factors profoundly influence the quantity of rabies virus produced in cell cultures, including the type of cell culture, the strain of virus, multiplicity of innoculum, the incubation temperature, the pH of the medium, and the protein supplement in the medium. These have been no definite report of a correlation between the stage of the cell cycle and susceptibility to the initiation of rabies virus infection. Fixed virus strains with a long history of in vitro passage appear to give the highest yields in cell cultures. Incubation temperature exerts a profound effect on the yield of rabies virus. Maximal virus titres are obtained in cultures incubated at temperatures of 32°C-35°C and in media of pH 7.6-8.0 (37).

(2) Morphological observations

Electron microscope observations of mammalian nervous tissues, salivary glands, and different cell cultures of neural and non-neural origin infected with rabies virus have revealed of virus particles that are frequently associated with characteristic matrices (Cytoplasmic inclusion bodies). In cell cultures and salivary glands,

numerous virus particles have been observed to originate from the cell surface as well as from intracytoplasmic membranes. The formation of matrices and nucleocapsids has also been shown in the absence of infectious virus. The cycle of virus replication may be as short as 6 hours after infection of cells (37).

(3) Virus-host cell interaction

Cell infected with rabies virus can generally be maintained in cell cultures for extensive periods of time without any noticeable cytopathic effect occuring. However, in some cell-culture systems, virus-host interactions can lead to cell destruction. In chronically infected cell cultures, cycles of high and low levels of infectious virus production and susceptibility to immunolysis seem to be mediated by the production of interferon. Virus propagated in tissue culture has proved a potent antigen for the immunization of animals when used either in the attenuated or inactivated state (37).

(4) Soluble antigens

Soluble complement - fixing viral antigens are present in rabies infected brains and cell cultures and can be separated from the virus particles by differential centrifugation, ultrafiltration, and chemical precipitation methods. The virion-depleted preparations are capable of inducing virus-neutralizing antibody and protecting animals against challenge. "Soluble" antigens are a mixture of fragmented and/or solubilized virus components derived from viral coat

and nucleocapsid structures. Solubilization of antigens from virus particles can also be obtained by treatment with detergents and other chemical compounds such as sodium deoxycholate, tributyl phosphate, saponin and others (37).

(5) Interferon

Experiments carried out to determine whether infection with rabies virus lead to the production of interferon and whether rabies virus is susceptible to interferon have given negative results in some laboratories and positive results in others (36). Replication of rabies virus can be inhibited in cell cultures treated with exogenous interferon. Live rabies virus and concentrated inactivated rabies vaccine are capable of inducing interferon both in vivo in experimental animals and in vitro in cell cultures. Experimental animals can be protected by treatment with synthetic interferon inducers and by administration of exogenous interferon. The demonstration in experimental animals that concentrated and inactivated rabies vaccine induces circulating interferon and that protection against challenge with street virus may be related to interferon induction seems to indicate a further possibility for prophylaxis, namely a treatment that combines interferon induction and immunization (37).

(6) Effect of metabolic inhibitors

Some inhibitors of DNA synthesis, such as arabinosyl cytosine, actinomycin D, or occasionally, rifampicin cause a decrease of

rabies virus yields in cell cultures.

Genetic markers are the properties of rabies viruses that have been traditionally used to characterize virus strains. Pathogenicity in different species and age groups, susceptibility of animals to peripheral as compared with CNS inoculation of virus, and variation in length of incubation period are the markers (37).

Immune response to rabies virus

Animals immunized against rabies may produce the following types of antibodies : virus-neutralizing, lytic (destroying rabies infected cells in the presence of complement, complement-fixing, and haemagglutination inhibiting antibody. Both lytic and neutralizing antibodies have been found in the immunoglobulin G (IgG) fraction of human and mouse sera (37).

Transfer of spleen cells from an animal inoculated intravenously 24 hours previously with rabies vaccine into another animal of the same inbred strain results in the formation of virus neutralizing antibodies by the recipient animal. Lymphoid cells derived from the spleens of normal mice will form virus neutralizing antibodies in cell cultures when exposed to rabies antigen. The presence of macrophages in this system is essential in order to activate the lymphoid cells for antibody production (37).

1.2 PATHOGENESIS

The virus is usually introduced by a bite wound, or scratch on

the skin inflicted by the rabid animal. The penetration can occur through intact mucous membranes and the digestive tract but not through intact skin (12, 17). Pathogenesis is believed to be essentially by neural transmission (12, 16, 36, 37). Blood-borne infection in nature is probably the exception rather than the rule (12, 36). When large amounts of virus are injected, blood-borne carriage is possible (17). Airborne infection of rabies from bats occasionally occurs (12, 16, 36, 37), and oral infection has been demonstrated in experimental animals (6, 37). In experimental animals the virus travels along the nerves centripetally at a rate of roughly 3 mm. per hour (17). Rabies virus ordinarily progresses from the site of exposure to the central nervous system via the peripheral nerves. Exceptions include young animals of highly susceptible species, such as the hamster, and animals whose resistance has been altered, for example by intracerebral trauma or shock. Once it reaches the CNS, the virus may then spread centrifugally to the peripheral nerves, infecting (and possibly multiplying in) the salivary glands, thymus, lymphnodes, pancreas, kidneys, and muscles (12, 36, 37). Virus growth in an organ may not indicate growth in the dominant cell type. Different viruses or different strains of virus have different cellular affinity. In the central nervous system the virus is found in the grey matter and appears to multiply only in the neurons (17). The susceptibility of an animal to rabies infection is influenced not only by the quantity of virus introduced into the animal but also by the site of the bite or injection, by the age of the animal, and by the

properties of the virus strain involved. The incubation period in the majority of human rabies cases is 3-8 weeks following exposure. In an animal model developed to give much the same periods, it was shown that the virus remains at or near the site of introduction for up to 2 weeks. Virus multiplication at the site of introduction may not be necessary to initiate infection (37).

Animals immunized before exposure to rabies virus are in general resistant to the infection if virus-neutralizing antibodies are present in their serum. On the other hand presence of virus-neutralizing antibody in the blood of an animal or human subject treated after exposure to rabies virus with immune serum and/or vaccine may not be a true indication of the protective effect of the postexposure treatment. Rabies virus is susceptible to the inhibiting effect of interferon. In experimental animals, interferon inducing products have been found to give protection when administrated soon after exposure (37).

1.3 VACCINE FOR IMMUNIZATION OF ANIMALS

The first practical vaccine for dogs was a phenolized rabbit brain product developed by Umeno and Doi. This type of vaccine was improved after Habel developed a standard potency test in mice. The results of the studies made by Johnson indicated that after a single dose of phenolized vaccine, dogs are protected for 1 year (12).

Koprowski and Cox developed a modified, living virus vaccine produced in chicken embryos (20). The strain of virus used in this vaccine was the Flury strain of 136 serial passages administered in

1-day-old chicks by the intracerebral route; subsequently it was adapted and passed 40 to 50 times in chicken embryos by Koprowski and Cox (12, 20). Experimental trials by Tierkel et al., Koprowski and Black, and Johnston et al. and field experiments, with this Low Egg Passage (LEP) chicken embryo vaccine showed that it has a high level of immunizing capacity in dogs, significantly superior to the phenolized and irradiated virus vaccines, and that the immunization lasted for more than 3 years following a singular muscular injection (12). Vienchange et al. were the first to cultivate rabies virus in cultures of mouse kidney cells (12). Kissling succeeded in growing and serially passing fixed and street rabies virus in hamster kidney cultures (12, 18). A vaccine prepared from virus grown in hamster kidney cultures showed good antigenic properties (11, 12). Since the last WHO* Expert Committee on Rabies met in June 1965, much new information has accrued, particularly in the fields of cell culture vaccines, such as the Modified Live Virus (MLV) vaccine utilize special cells of animals, either primary cell cultures as selected continuous cell lines (37).

Several types of Rabies vaccines listed in Table 1 are of established value and have been used throughout the world. Vaccines prepared from the Low Egg Passage (LEP) and from the High Egg Passage (HEP) flury strain of the Modified Live Virus (MLV) infected in chick embryo origin (CEO) and the inactivated nervous tissue vaccines prepared from the brains of goats or sheep infected with fixed strains of rabies virus, have been found effective for pre-exposure use under

* WHO = World Health Organization

TABLE 1

Types of Vaccines for Use in Animals (37)

Vaccine	For use in	Regimen recommended			
		Primary immunization			Booster dose
		dose(s)	animal's age	route	
LIVE VIRUS					
Chick embryo					
low egg passage Flury (LEP Flury)	dogs	1	3 months & 1 year[b]	i.m.	every 3 years
high egg passage Flury (HEP Flury)	dogs	1	3 months	i.m.	annually
	cats	1	3 months	i.m.	annually
	cattle	2 (1 month apart)	as required	i.m.	annually
Kelev [a]	dogs	1	3 months & 1 year	i.m.	annually
	cattle	2	as required	i.m.	annually
Cell culture					
canine kidney (HEP Flury)	dogs	1	3 months & 1 year[b]	i.m.	every 3 years
	cats	1	3 months	i.m.	annually
	cattle	2 (6 weeks apart)	as required	i.m.	annually
porcine kidney (ERA)	dogs	1	3 months & 1 year[b]	i.m.	every 3 years
	cats	1	3 months	i.m.	annually
	cattle	1	4 months	i.m.	every 3 years
	horses	1	4 months	i.m.	every 2 years
	sheep, goats	1	4 months	i.m.	annually
chick embryo fibroblasts (LEP Flury)	dogs	1	3 months & 1 year[b]	i.m.	every 3 years
hamster kidney (LEP Flury)	dogs	1	3 months & 1 year[b]	i.m.	every 3 years
hamster kidney [a] (Vnukovo-32)	dogs	1	3 months & 1 year	i.m.	annually
	cats	1	as required	i.m.	annually
	cattle	1	as required	i.m.	annually
	horses	1	as required	i.m.	annually
	sheep, goats	1	as required	i.m.	annually
INACTIVATED					
Nervous tissue					
suckling mouse brain (fixed virus)	dogs	1	3 months & 1 year	i.m.	every 2–3 years
	cats	1	3 months	i.m.	annually
	cattle	1	as required	i.m.	annually
caprine, ovine (fixed virus)	dogs	1	3 months	i.m. or s.c.	annually
	cats	1	3 months	i.m. or s.c.	annually
	cattle	1	as required	i.m. or s.c.	annually
Cell culture					
hamster kidney (fixed virus)	dogs	2	3 & 4 months	i.m. or s.c.	annually
	cats	2	3 & 4 months	i.m. or s.c.	annually
	cattle, horses	2	as required	i.m. or s.c.	annually
	sheep, goats	2	as required	i.m. or s.c.	annually
porcine kidney [a] (LEP Flury)	dogs	2	3 & 4 months	i.m. or s.c.	every 1–2 years
	cats	2	3 & 4 months	i.m. or s.c.	every 1–2 years
	cattle	2	as required	i.m. or s.c.	annually
	horses	2	as required	i.m. or s.c.	annually
	sheeps, goats	2	as required	i.m. or s.c.	annually

[a] These vaccines have not been included in the comparative immunization studies on which the recommendations for the other vaccines have been based. Recommendations for these vaccines are based on the best information available to the Committee, according to the type of vaccine described.

[b] In mass vaccination programmes the schedule of primary immunization can consist of only 1 inoculation given each year to all dogs between 3 months and 1 year of age.

field conditions in large numbers of animals of various ages and breeds. They contained large quantities of the tissue which have been responsible for undesirable reactions in many animals, especially when repeated injections have been administered. The tissue-culture vaccines and suckling mouse-brain (SMB) vaccine, the newer vaccines have been developed to provide safer but equally potent or more potent products for use in animals. The strains of virus used in the production of MLV tissue culture vaccines have included the established LEP or HEP strains, the ERA strain, and the Vnukovo strain. Fixed-rabies virus strains have been utilized in the production of SMB vaccine. These vaccines have been used in millions of animals during the past few years (37). The live-virus rabies vaccines grown in tissue culture had less measurable infective rabies antigen than the LEP-CEO vaccine. Regardless of the amounts of virus in the vaccine tested, evidence was provided that all the live-virus vaccines either multiplied or remained viable in dogs over a long period. The median antibody titers were higher at the second year than at the first year of the dog vaccinated with MLV vaccine, but the titers were lower at the second year than at the first year of the dogs vaccinated with an inactivated vaccine. The guinea pig potency test is satisfactory for measuring the immunogenicity of MLV canine rabies vaccines (31).

Recently two types of modified live virus vaccine studies conducted in USA in adult dogs with MLV tissue-culture and LEP chick embryo vaccines. MLV protested dogs for 3 years as well as did the low-egg passage with a single dose of vaccine. A single injection of

the SMB vaccine protected of the dogs for 1 year and 80% of them for 3 years. Best results also were obtained with an experimental inactivated and purified brain-tissue vaccine. A single dose of the inactivated hamster kidney-tissue-culture was not as effective as the MLV cell-culture or chick-embryo types for either 1 or 3 years duration (37). Recent comparative vaccine studies conducted in Latin America in adult cattle with MLV Tissue-culture, HEP chick-embryo vaccines and the inactivated SMB vaccine showed that a single dose provided a duration of immunity of at least 1 year. The ERA vaccine provided 3 years protection (37).

The ERA strain of rabies vaccine produced in pig kidney tissue culture is the newly developed Tissue-culture vaccine for animals, with improvements in the safety and efficiency and provided protection for 3 years. Dr. M.K. Abelseth reported, on the use of ERA vaccine produced in pig kidney tissue culture for the immunization of domestic animals against rabies, that it was highly antigenic in cats, dogs, goats, cattle, and horses as measured serologically and by challenge with field virus, and described the pathogenicity and antigenicity for laboratory animals. Duration of immunity has been demonstrated for 3 years in cattle, and 2 years for dogs and horses. A uniform dose of 2 ml is recommended regardless of animal size (1, 3).

The ERA strain of rabies virus as propagated in swine kidney cells is pathogenic to mice, guinea pigs, and hamsters by the intracerebral route. The pathogenicity to these speices shown by the intramuscular route is low. The trial in domestic animals such as

dogs, cats, and cattle has been found to be safe by the intramuscular route (1). The oral vaccination of foxes against rabies by dropping 1 ml of a high titered attenuated rabies virus (ERA strain rabies virus grown on BHK - 21 cells which the virus titer was $10^{-7.0}$LD 50/0.03 ml) on the tongue and buccal mucosa. The animals developed serum neutralizing antibodies and resisted challenge. After challenged with the street virus suspension from a naturally infected fox from northern New York, four of the five controls died, while all the vaccinates remained in good health during the three months observation period. The incubation periods of the four control foxes were 24, 25, 25, and 27 days (6).

An antigenic extinction trial in cats showed that the ERA rabies vaccine had superior antigenic properties. Dogs and cats on a duration of immunity study of ERA rabies vaccine were challenged with fox salivary gland "street" rabies virus. The results of this challenge show a duration of immunity of five years in dogs and four years in cats. Vaccination of dams in late pregnancy with ERA rabies vaccine resulted in transference of maternal antibody to the newborn, in both cattle and dogs. Maternal antibodies in most of the puppies tested persisted until ten weeks of age (24).

Research studies on the vaccine have extended over a period of more than nine years (from 1963 to date). The information contained in the following table represents a summary of the studies reported in the scientific articles cited. The following four points were consistent throughout :

1. The modified live virus, porcine tissue culture origin, ERA Strain was used.
2. Dosage 2 ml. In small animals the vaccine may be used with only 1 ml of diluent per dose.
3. Route of administration : Intramuscular.
4. Challenge virus consisted of a standardized pool of virulent rabies virus obtained directly from the salivary glands of rabid foxes.

Dogs and cats trials

Dogs trials : Table 2 gives a summary of nine separate trials where dogs were vaccinated at two months of age or older and challenged after a holding period of 40 days to 63 months.

TABLE 2

Summary of Dog Trials

Vaccination, challenge and S-N* results (8)

	Challenge Results		
Interval Between Vaccinated & Challenged	Vaccinated Survived/ Challenged	Controls Survived/ Challenged	S-N Results
40 Days	8/9	0/9	<5 to >25
49 Days	7/7	3/15	Not reported
51 Days	8/8	2/15	Not reported
60 Days	66/67	0/15	<5 to 25
12 Months	25/25	0/10	<5 to 19
24 Months	22/22	0/9	<5 to 12
36 Months	6/6	0/5	0 to 25
48 Months	6/10	0/9	5 to 17
63 Months	13/14	5/14	0 to >25
Total	161/168	10/101	

* S-N Results = Serum Neutralizing Results.

This trial (Table 3) was designed to determine how far the vaccine could be diluted and still give protection in dogs. The vaccine, even when given at a dilution 1/1000, is capable of producing in dogs a significant level of antibodies and giving protection against a massive challenge dose of field virus 41 days after vaccination.

TABLE 3

Vaccine Dilution Trial in Dogs

Challenge and S-N results (8)

Vaccine Dilution	Survived/ Challenged	S-N Results			
		<5	5	25	>25
Undiluted	4/4		1		3
1/10	4/4			2	2
1/100	4/4			4	
1/1000	3/4	2		1	1
Controls	0/4	4			

Cats trials : Table 4 shows the results of three trials where cats were challenged after a vaccination period of 71 days to 55 months.

TABLE 4

Summary of Cat Trials

Vaccination, challenge and S-N results (8)

	Challenge Results		
Interval Between Vaccinated & Challenged	Vaccinated Survived/ Challenged	Controls Survived/ Challenged	S-N Results
71-85 Days	36/38	2/14	Not reported
28 Months	9/10	0/11	0 to >25
55 Months	8/8	1/10	0 to >25

TABLE 5

Vaccine Dilution Trial in Cats

Challenge and S-N results (8)

Vaccine Dilution	Survived/ Challenged	S-N Results			
		<5	5	25	>25
Undiluted	10/10		1	8	1
1/10	9/9		3	6	
1/100	8/10	5	1	4	
1/1000	4/10	10			
Controls	3/11	11			

Cattle, Sheep, Goats and Horses Trials :

Cattle trials : Table 6 gives a summary of seven separate trials where cattle were vaccinated and subsequently challenged, after holding periods of 30 days to 48 months. In the tests, all the vaccinated animals withstood the challenge, whereas all the controls died. Detectable antibody levels were present 48 months after vaccination.

TABLE 6

Summary of Cattle Trials

Vaccination, challenge and S-N results (8)

Interval Between Vaccinated & Challenged	Challenge Results		S-N Results
	Vaccinated Survived/ Challenged	Controls Survived/ Challenged	
30 Days	4/4	0/5	>25
35 Days	4/4	0/4	5 to >25
10 Months	10/10	0/5	>25
18 Months	10/10	0/5	<5 to 55
24 Months	10/10	0/5	<5 to 69
36 Months	10/10	0/5	<5 to 16
48 Months	6/6	0/5	2 to 25
Total	54/54	0/34	

This trial (Table 7) was designed to determine if the vaccine could be diluted and still give protection in cattle. Complete protection was found at 1/100 dilution of vaccine, with a significant antibody level in all dilutions except 1/1000 forty-one days after vaccination.

TABLE 7

Vaccine Dilution Trial in Cattle

Challenge and S-N results (8)

Vaccine Dilution	Survived/ Challenged	S-N Results			
		<5	5	25	>25
Undiluted	4/4		1	2	1
1/10	4/4				4
1/100	4/4		1	1	2
1/1000	1/4	4			
Controls	0/4	4			

Sheep trials : This trial (Table 8) was designed to determine how far the vaccine could be diluted and still give protection. Protection was found in all dilutions up to 1/1000, although antibodies were not present beyond the 1/1000 dilution of vaccine one month after vaccination.

TABLE 8

Vaccine Dilution Trial in Sheep

Challenge and S-N results (8)

Vaccine Dilution	Survived/ Challenged	S-N Results			
		<5	5	25	>25
Undiluted	5/5	1	1	3	
1/10	5/5	2	1	2	
1/100	5/5		2	2	1
1/1000	4/5	5			
Controls	1/7	7			

Goats trials : This trial (Table 9) was designed to determine the effectiveness of the vaccine in goats. The eleven goats that were vaccinated and challenged were immune and the four controls susceptible.

TABLE 9

Summary of Trial in Goats

Vaccination, challenge and S-N results (8)

Interval Between Vaccinated & Challenged	Challenge Results		S-N Results
	Vaccinated Survived/ Challenged	Controls Survived/ Challenged	
56 Days	11/11	0/4	5 to 39

Horses trials : Table 10 gives a summary of two trials in which horses were vaccinated and challenged after 94 days and 24 months. In the two trials, all the horses withstood the challenge.

TABLE 10

Summary of Horse Trials

Vaccination, challenge and S-N results (8)

Interval Between Vaccinated & Challenged	Challenge Results		S-N Results
	Vaccinated Survived/ Challenged	Controls Survived/ Challenged	
94 Days	7/7	0/2	5 to 16
24 Months	7/7	0/3	5 to 19

The summary of the information for using the rabies vaccine, ERA strain are as followed :

1. Safety in domestic animals : this vaccine has been found to be safe for dogs, cats (pets) ; cattle, sheep, goats and horses (Farm animals).

2. Efficacy, this vaccine has been found to be effective in the laboratory as shown by actual vaccination and challenge trials, and in the field in areas where rabies is endemic.

3. Duration of immunity, laboratory trials have shown protection for at least the following periods :

Animal Species	Interval Between Vaccination and Challenge
Dogs	63 Months
Cats	55 Months
Cattle	48 Months
Horses	24 Months

The antibody levels following vaccination have been measured by a serum neutralizing (S-N) test and indicate a significant level even after 48 months from date of a single vaccination in cattle.

4. Vaccine dilution trials in cattle, cats, dogs, and sheep have demonstrated a high level of protection. This indicates an added margin of effectiveness in the field.

5. Uniform dosage 2 ml regardless of animal species. In very small animals such as kittens, vaccine may be reconstituted with 1 ml of diluent per dose instead of the usual 2 ml.

6. Route of administration, Intramuscular.

7. Age to be vaccinated

Cattle, sheep, horses and goats : 4 months of age and over

Dogs and cats : 2 months of age and over

8. Reactions; the vaccine is made in a tissue culture system,

which gives a product low in foreign protein and thus reduces the danger of reaction (8).

1.4 VIRUS STRAINS

ERA strain of SAD Virus (35-45 passages), porcine cell culture is used as seeded virus.

Development of ERA strain

1. The original virus from a rabid dog was obtained in 1935 from Montgomery, Alabama as a mouse adapted isolate (it had been passed many times in mice). It has been in use as a challenge virus for testing Semple vaccine in mice.

2. Four further passages were made in mice. The virus was given the name SAD_4 (Street - Alabama - Dufferin).

3. The SAD_4 was adapted to a hamster kidney tissue culture system. Twenty-five passages were made in this system. The virus was designated $SAD_4\ HK_{25}$ (8).

In 1958, Kissling has reported the successful propagation of rabies virus in hamster kidney cell cultures (1, 18). Subsequently Kaplan, Forsek and Koprowski and Fenje also reported the growth of rabies virus in hamster cell culture (2, 10, 11, 20).

In this experiment the seed virus was a suspension of mouse brain representing the 30th passage of strain SAD in an alternate mouse brain hamster kidney sequence. The infecting dose 1000 mouse

LD_{50} units, was added to the nutrient medium in contact with the cell monolayer and the culture was incubated at 37°C, with replacement of the fluid in the dialysis tube every 4 days. Samples of the culture fluid in contact with the cells were removed at intervals and titrated immediately by intracerebral innoculation of white mice. A summary of the results is given in Table 11 (11).

TABLE 11

Growth of rabies virus strain SAD in hamster kidney tissue cultures (dialysis tube method) (11).

Serial No. of sample	Time of removal of sample	Infectivity of culture fluid (mouse brain LD_{50} doses/ml)
1	At start	$10^{4.47}$
2	3 hours	$10^{3.17}$
3	1 day	$10^{2.47}$
4	2 days	$10^{3.47}$
5	3 "	$10^{4.65}$
6	4 "	$10^{5.50}$
7	13 "	$10^{6.21}$
8	20 "	$10^{6.21}$
9	23 "	$10^{7.00}$
10	29 "	$10^{7.00}$
11	36 "	$10^{6.00}$
12	46 "	$10^{5.47}$
13	53 "	$10^{5.23}$
14	62 "	$10^{2.70}$
Control *		
1	At start	$10^{4.47}$
2	3 hours	$10^{4.47}$
3	1 day	$10^{1.95}$
4	2 days	Not infective
5	3 "	"
6	4 "	"

* The control tubes were set up in the same manner as those of the test except that they contained no cell monolayer.

At a later stage of these investigations the SAD strain became better adapted to growth in hamster kidney cells and serial transfer in these cells was at length accomplished. The dialysis tube technique was continued.

The results of one serial cultivation experiment are presented in Table 12.

TABLE 12

Rabies virus strain SAD maintained by serial transfers in cultures of hamster kidney cells grown in dialysis tubes (11)

Number of transfers in hamster kidney cells	Period of cultivation of cells after infection (days)	Infectivity of culture fluids (LD_{50} doses/ml)
2nd	12	$10^{5.0}$
2nd	19	$10^{7.0}$
3rd	8	$10^{4.7}$
3rd	15	$10^{4.7}$
4th	8	$10^{5.7}$
5th	6	$10^{4.0}$
5th	13	$10^{4.3}$
5th	30	$10^{3.7}$
6th	7	$10^{4.7}$
6th	11	$10^{5.0}$
6th	18	$10^{5.0}$
7th	8	$10^{5.0}$
7th	15	$10^{4.0}$

The pathogenicity of the virus was not appreciably altered as the result of adapting it to hamster kidney cells. Mice inoculated intracerebrally with the culture virus developed encephalitis and died 6 or 7 days after injection. In the case of rabbits, the incubation period following intracerebral injection was increased to 12 to 13 days. The brain tissues of mice and rabbits injected with the adapted strain were examined by the usual methods of histopathology. There was the usual picture of a viral encephalitis. Negri body inclusions were not found, but many of the neurons contained finely granular acidophil material diffusely situated in the cytoplasm. The appearance of the hamster kidney cells in tissue culture was studied by the flying cover glass technique. It was surprising to find very few pathological changes, even in those cells that had been exposed to the virus for periods as long as 3 weeks (11).

4. SAD_4HK_{25} was passaged ten times in chick embryo via the yolk sac route (8). The rabies virus obtained from Dr. Paul Fenje (11), having a titer of 10^{-3} per 0.03 ml, was inoculated into seven-day incubated eggs via the yolk sac route. Propagation of the virus in egg embryo was shown in that the titer after eight passages was over 10^{-2} per 0.03 ml (2).

5. It was next adapted to pig kidney tissue culture and was passaged on additional six times. At this stage, it became attenuated to the point that it was safe for various species of animals. It was designated as ERA, after the names of the principal investigators,

that are Miss Evelyn Gayner (E), Mr. Alec Rockitnicki (R) and Dr. M.K. Abelseth (A) (2, 8).

6. Passage was continued, and the 35-45th passages were used for production of the new ERA strain vaccine in a tissue culture system (8).

7. Continueing studies have shown that the 85th passages retains immunogenicity for dogs and cattle (8).

Dr. M.K. Abelseth reported the successful adaptation of rabies virus to pig kidney cell monolayers. He produced the rabies vaccine for the immunization, not only of dogs, but especially of cattle and sheep, which are the main victims of sylvatic rabies (2).

Results of the tissue culture passages are shown in Table 13.

TABLE 13

Rabies Virus Titres in Pig Kidney Cell Cultures* (2)

Passage No.	6th Day	7th Day	9th Day	10th Day	14th Day	21st Day
1	Neg.		Neg.			
2		Pos.			Pos.	
3		Pos.			Pos.	
4		Pos.			Pos.	
5		$10^{-1.1}$		$10^{-2.4}$	$10^{-2.3}$	
6		$10^{-2.1}$		$10^{-3.3}$	$10^{-3.2}$	
7	$10^{-2.4}$		$10^{-2.3}$		$10^{-3.2}$	
8		$10^{-1.4}$		$10^{-3.2}$	$10^{-3.0}$	
9		$<10^{-3.0}$		$10^{-3.2}$	$<10^{-4.0}$	
10		$>10^{-4.0}$		$10^{-3.3}$	$10^{-4.4}$	
11		$10^{-4.3}$		$10^{-4.4}$	$<10^{-5.0}$	$10^{-4.4}$
12		$>10^{-5.0}$		$10^{-4.5}$	$10^{-5.5}$	$10^{-4.3}$
13		$10^{-4.8}$		$10^{-4.3}$	$10^{-4.2}$	$<10^{-4.0}$
14		$10^{-4.5}$		$10^{-4.5}$	$10^{-6.0}$	$<10^{-4.0}$
15		$10^{-4.0}$		$10^{-4.4}$	$<10^{-5.5}$	$<10^{-4.0}$
16		$10^{-5.6}$		$10^{-4.6}$	$10^{-5.4}$	
17		$10^{-4.4}$		$10^{-4.3}$	$<10^{-4.0}$	
18	$<10^{-3.0}$		$10^{-5.6}$		$>10^{-6.0}$	$10^{-3.0}$
19		$10^{-5.0}$		$<10^{-4.0}$	$10^{-5.5}$	
20		$10^{-5.0}$		$>10^{-6.0}$	$10^{-5.4}$	
21		$10^{-4.0}$		$<10^{-4.0}$		
22		$10^{-4.8}$		$10^{-5.6}$		
23		$10^{-4.2}$		$10^{-5.4}$	$10^{-5.0}$	
24		$10^{-5.0}$		$10^{-5.5}$	$10^{-4.0}$	
25		$10^{-5.5}$		$10^{-4.3}$	$10^{-4.0}$	
26		$10^{-4.8}$		$10^{-4.2}$	$10^{-3.0}$	
27	$10^{-3.5}$		$10^{-4.8}$			
28		$10^{-4.5}$		$10^{-5.0}$		
29		$10^{-4.3}$		$10^{-5.0}$		
30		$10^{-4.8}$		$10^{-4.5}$		

* Per 0.03 ml in 15 gram mice intracerebrally.

This table demonstrates that rabies virus could be grown in pig kidney cells and good titers were obtained. In carrying out further tissue culture passage the virus was immunogenic and nonpathogenic to several species of animals. As passages were continued, less incubation time was necessary to reach maximum titers. At the seventh passage 14 days were required, whereas at the twenty fifth passage, maximum titer was obtained at seven days. The virus multiplies quite rapidly since the replacement of fluid on infected cells results in a titer usually equal to that present before fluid change. At least three times of fluid could be harvested from the one monolayer culture (2).

1.5 RABIES IN THAILAND

Rabies is a considerable health problem as an enzootic disease in many parts of the world. According to a world-wide survey of the World Health Organization in 1962-1963, rabies was present in 69 countries. In general, the chief natural hosts of rabies are canines, however, felines and other wild animals such as mongoose, raccoons, skunks, rats and bats can also be the sources of infection (33).

Rabies is of a major importance among domestic animals both in rural and urban areas in Thailand. SEATO Medical Research Laboratory (SMRL) reported cattle, monkey and the dog-faced bat *Cynoterus branchyotis* to be dangerous reservoirs of rabies infection in Thailand. The percentage of virus recovery was highest in Indian Bandicoot rat - *Bandicota indicus* (7.9 percent) (10, 33). The canine rabies are

essential for further planning of both prevention and control in Thailand. Dogs are the most important source of rabies in man, whereas cats usually play a much less important role. At the Queen Saovabha Memorial Institute, the number of rabid dogs was 1,326 out of 1,707 specimens examined in the average of 1970 to 1975 (Table 14). At the SMRL, 629 samples of dogs brains were examined in the average of 1970-1972 with 45% positive for rabies (Table 15). At the Siriraj Hospital Laboratory, about 20 cases of rabid animals are diagnosed each year (33).

TABLE 14

Number of dog's brain examined at the Queen Saovabha Memorial Institute in 1970-1975 (34)

Year	Number Examined	Positive for rabies (Microscopic examination and animal innoculation)
1970	1,047	794
1971	1,358	1,155
1972	1,810	1,472
1973	1,866	1,405
1974	1,875	1,456
1975	2,290	1,677
Average	1,707	1,326

TABLE 15

Number of dog's brain examined at the SMRL (34)

Year	Number examined	Positive for rabies (FRA* and animal inoculation)
1970	426	199
1971	584	294
1972	702	337
1973	805	308
Average	629	285

* FRA = Fluorescent Rabies Antibody

Most of the rabid animals in Bangkok are dogs. However, it should be noted that many of rabid animals are not accounted to these tables because they may be killed or die spontaneously of the infection unrecognized or undiagnosed. And also the actual numbers of dogs and cats in the Bangkok Metropolis and the other provinces of Thailand are not yet known.

TABLE 16

Number of dogs caught destroyed and vaccinated against rabies at the dog-pound by the Health Authorities of Bangkok Municipality (34).

Year	Caught	Destroyed	Vaccinated
1970	38,805	36,233	10,506
1971	40,563	37,864	11,945
1972	48,670	44,686	7,229
1973	51,626	45,121	7,603
1974	48,350	42,423	5,406
1975	33,404	28,437	4,260

According to the survey program by the SMRL 3 percent positive cases were found among the stray dogs (32).

In order to substantiate the situation that the finding is most likely representing the figures of rabid dogs in Bangkok Metropolis, references are made to the following data : (34)

1970

Number of dogs (mostly stray dogs) caught by the Health Authorities of Bangkok (3 to 4 percent rabid)	38,805
Number of dogs vaccinated against rabies at the Health Authorities in Bangkok	10,506
Number of rabid dogs as confirmed by laboratories techniques	1,000
Number of human deaths of rabies in Bangkok	37
Number of persons vaccinated against rabies at the Queen Saovabha Memorial Institute (Semple vaccine)	7,797
Number of rabies vaccine (duck embryo) imported approx. (vials)	5,000

As mentioned above, the animal population census is very important for dog control. A great number of stray dogs in Bangkok have been caught and destroyed each year by the Health Authority of the Metropolitan Municipality, as a measure to control rabies among animals. A simple method, using contraceptive injections in order to

reduce the population in dogs has been introduced for the first time in Control Program since 1975. To complete the control operation, vaccination against rabies must be done under the mobile units from place to place.

Vaccination in domestic animals, especially in dogs has been encouraged for the prevention of rabies in the past few years. There are two types of imported animal vaccines in Thailand the Flury Low Egg Passage (LEP) of chick embryo origin and the Flury High Egg Passage (HEP) of tissue culture origin. Unfortunately the vaccine produced locally in Thailand is only the phenolized sheep brain type and not yet enough for the whole population. This work has been done since 1975 on the production of Modified Live Virus Rabies Vaccine Procine Tissue Culture origin, ERA strain that will be effectively and sufficiently in the Rabies control operations.