

PREPARATION OF RABIES MONOCLONAL ANTIBODIES AS
A DIAGNOSTIC TOOL FOR RABIES STRAIN
DIFFERENTIATION

THESIS SUBMITTED
UNIVERSITY OF MUMBAI

FOR THE DEGREE OF
MASTER OF SCIENCE
IN
MICROBIOLOGY

SUBMITTED BY
SWAPNAJA PRAKASH GHADIGAONKAR

UNDER THE GUIDANCE OF
DR. (MRS.) R. A. DESHMUKH

DEPARTMENT OF VIROLOGY
HAFFKINE INSTITUTE FOR TRAINING, RESEARCH & TESTING
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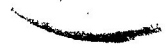
STATEMENT BY THE CANDIDATE

As required by the University Regulation No.2190, I wish to state the work embodied in this thesis titled “**Preparation Of Rabies Monoclonal Antibodies As A Diagnostic Tool For Rabies Strain Differentiation**”, forms my own contribution to the research work carried out under the guidance of **Dr. (Mrs.) R. A. Deshmukh** at the Department of Virology, Haffkine Institute, Parel, Mumbai 400 012. This work has not been submitted for any other degree or this or any other University. Whenever references have been made to previous works of others, it has been clearly indicated as such and included in the Bibliography.


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Dedicated Lovingly to My Family

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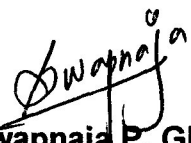
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INTRODUCTION

PREPARATION OF RABIES MONOCLONAL ANTIBODIES AS A DIAGNOSTIC TOOL FOR RABIES STRAIN DIFFERENTIATION

“Dogs suffer from three diseases: rabies, quinsy and sore feet. Rabies drives the animal mad, or any animal whatever, excepting man, will take the disease if bitten by a dog so afflicted; the disease is fatal to the dog itself and to the animal it may bite, man excepted.”

– Aristotle

Historia animalia VIII, 22, 604a (circa 4th century BC)

The above quote from Aristotle's “The Natural History of Animals” during the fourth century BC is a blatant example of the disbelief that man is superior to other animals. The irony of this quote and the current scenario of rabies as a threat to humans and all the mammals cannot be ignored.

Rabies is an acute, neurological disease caused by infection of rabies virus involving central nervous system. There are very few viruses as successful as rabies virus which had survived for millennia in most parts of the world. It can replicate in all infected animals, where the resultant disease is almost invariably fatal. Lack of awareness, low level of political commitment to rabies control, and failure to recognize and correlate the clinical features with the laboratory findings, contribute to deaths. The virus ensures its own survival by causing the afflicted host to find another host for it. Therefore rabies virus continues to be an important threat for humans and veterinary animals because of its extensive reservoirs in diverse species of wildlife. However :

strong scientific grasp on the transmission and progress of the disease has allowed many nations to initiate public health campaigns that have nearly wiped out the incidence of human rabies throughout the developed world. Survival of patients, though infected, relies on early innate and adaptive immunity developed after vaccination (1, 2).

Since animal bite is the primary route of rabies virus transmission to humans, the epidemiology of human rabies is a direct reflection of the regional animal reservoir for this virus (3). The epidemiology of rabies has changed over recent years, as this disease has been brought under control or eliminated in many terrestrial animal species in Europe and North America. However, it still remains endemic in many countries in Africa and Asia, largely as a result of financial limitations and a poor medical/veterinary infrastructure (4, 5). A large number of rabies virus variants have now been characterized, and their distribution and animal hosts have become known. According to World Health Organization (WHO), more than three billion people are at risk for rabies in over 85 countries and territories worldwide, and about 50,000–60,000 human deaths from rabies are estimated to occur annually (6). Although all age groups are susceptible, 30–60% of these cases being seen in young children. As per WHO weekly epidemiological record (2002), more than 99% of all human deaths from rabies occurred in Asia, Africa and South America. India alone reported 30,000 deaths annually (7). Asia carries a larger part of the public health burden of rabies with an estimated 31,000 deaths, although the estimate for Africa (24,000 deaths) is much greater than initially believed. Even though effective vaccines for post-exposure prophylaxis (PEP) are

available, only 10 million individuals actually receive rabies PEP each year (6). Rabies is present uniformly in all states of India except in the Andaman Nicobar and Lakshadweep islands. In neighbouring countries such as Thailand, Sri Lanka and Philippines rabies deaths have been drastically reduced (8). In 2003, a National Survey by the Association for the Prevention and Control of Rabies in India estimated 18,500 human deaths from rabies in the country each year, out of which 89% had not taken PEP. In India 1.0 million patients receive post-exposure treatment annually (9, 10). In India rabies is not a notifiable disease, figures of human deaths and livestock losses caused by rabies are poorly documented. There are very few reports from India recording the number of cases of rabies and animal bites. In most countries of Africa, Asia, Latin America, and the Middle East, dogs are found to be the main hosts and are responsible for most of the human rabies deaths that occur worldwide.

The most common mode of transmission in man is through the bite of a rabid animal or the contamination of scratch wounds by virus-infected saliva. However, other routes have been implicated in the past, such as through mucous membranes of the mouth, conjunctiva, anus and genitalia. Infection by aerosol transmission had been demonstrated in experimental animals and has been implicated in human infection in rabies-infected bat caverns and in several laboratory accidents (6). Man to man transmissions by transplantation of infected corneas were reported in five instances. Recently three cases of human rabies after organ transplantation occurred in America (11). In addition, the infection may occur from infected lactating mother to her child.

The rabies virus is RNA virus which has been classified as a member of the genus *Lyssavirus* within the Rhabdoviridae family. The *Lyssavirus* genus consists of seven serotypes on the basis of serological and antigenic relationships. Serotype one consists of 'classical' rabies viruses, notably the 'street' (wild) and vaccine strains viz. Pitman-Moore, Paris strain, Flury-HEP Flury-LEP etc. Serotypes 2, 3, 4, 5, 6 and 7 consist of several rabies-related viruses. They share high levels of amino acid and nucleotide sequence homology with rabies virus and cause a clinical disease indistinguishable from rabies encephalitis. Specific identification is performed by using monoclonal antibodies (MAbs) and genetic typing. Further, animals protected against classical rabies virus are not necessarily immune to infection with the rabies-related viruses due to antigenic difference between them (12).

Each virus is bullet-shaped and approximately 180 nm long and 75 nm wide. The lipid bilayer membrane is coated with numerous 10 nm spikes composed of glycoprotein. Inside the membrane is a lining of matrix protein and a core containing the ribonucleoprotein complexes. The genome of the rabies virus is made up of approximately 12,000 nucleotides of single-stranded RNA. This contains five genes which code for each of the five viral proteins.

- RNA dependent RNA polymerase (L protein) (180 KD).
- Glycoprotein (G), exists in two forms (G I) (65 KD) and (G II) (70 KD).
- Nucleoprotein (N) (57 KD).
- Phosphoprotein (M1), exists in two forms (M1 I) (38 KD) and (M1 II) (41KD)

- Matrix or membrane protein (M2) having two conformations (M2 I) (24KD) and (M2 II) (25 KD).

These antigens carry one or more antigenic sites which are serotype specific but cross-reactions are seen with some rabies related viruses. M1, M2, and L proteins are considered as potential antigens, whereas G protein is responsible for induction of virus neutralizing antibodies, which are responsible for the protection of animals against challenge virus strain. Glycoprotein also contains determinants of virulence and is responsible for the specific serological reactions of the virus. Antigenic difference among strains of rabies virus can be easily detected using a panel of monoclonal antibodies (MAbs) directed against the G protein of the virion. The N protein on the other hand is a group protein which cross-reacts with antiserum produced from the rabies related viruses (13). Monoclonal antibodies directed against the nucleocapsid protein have been used to differentiate and classify rabies wild isolates into types of lyssaviruses (14). Antigenic difference between the rabies strains may cause vaccine failure (15).

Previously, no specific diagnostic test existed to differentiate vaccine strains from street strains and rabies from rabies related virus strains. Now the specific identification of rabies isolates can be done by using MAbs and genetic typing (16). The immunofluorescence assay using fluorescent tagged MAbs is a widely used technique for the rabies diagnosis. The molecular methods include Polymerase Chain Reaction (PCR), Strain specific reverse transcription PCR (RT-PCR), Nucleic acid sequence-based amplification (NASBA) and Restriction fragment length polymorphism (RFLP) analysis or multiplex PCR (17, 18). However, these methods discriminate only few

variants; further, these methods are expensive and require specialized technical facilities and expertise. Systematic typing by genetic methods is time consuming and expensive. Thus it was found that, routine application of antigenic differentiation, employing a limited panel of monoclonal antibodies in an indirect immunofluorescence technique is more practical, sensitive specific, rapid and easy to perform (16).

In 1975, Kohler and Milstein reported successful production of MAbs using the hybridoma technique (19). The technique involves production of hybridomas by the fusion of mouse B lymphocytes and myeloma cells. B lymphocytes from immunized mouse have the capacity to produce antibodies against the antigen used, whereas myeloma cells are malignant cells which are used to proliferate rapidly and secrete larger amounts of antibodies. A solution of MAbs consists of identical molecules with identical specificity and affinity for antigen. A solution of antibody derived from polyclonal serum in contrast contains a mixture of antibodies. There will be antibodies against several epitopes of the antigen of interest. Even among antibody against the same epitope, there will be antibodies of differing affinity and different subclass. Monoclonal antibodies have the advantage that once you have isolated a stable clone, you can produce identical antibodies indefinitely for years. Monoclonal antibodies are useful research tool in analysis of structure and function of microorganisms and viruses and have become powerful reagents in the diagnosis of infections (20).

In 1978, Wiktor and Koprowski first described production of MAbs directed against the rabies virus. Monoclonal antibodies were produced by hybridoma

of fused mouse myeloma cells and splenocytes from mice immunized with either rabies virus or rabies related viruses. These hybridoma-secreted MAbs were directed against G and N protein of rabies virus. These antibodies displayed specific reactivity patterns which were used to characterize and classify rabies and rabies related viruses into groups corresponding to antigenic determinants (21). In addition vaccine strains of rabies were differentiated from wild isolates.

To date, several *Lyssavirus* isolates from humans, domestic animals, and wild animals in Africa, America, Asia and Western Europe have been compared using MAbs (22). Nowadays MAbs directed to the surface glycoprotein, nucleoprotein, phosphoprotein and matrix protein are used to characterize rabies viruses. The different strains of rabies viruses show marked differences in their reactivity with hybridoma antibodies. Previous application of traditional epidemiologic methods to such outbreaks was limited since rabies viruses were considered antigenically similar regardless of their geographic or specific origin. However the availability of MAbs which distinguish closely related natural rabies variants can provide potential clues to the origins of epizootics. Also these can be used to detect the cause of vaccine failure cases.

The greater role for rabies MAbs may prove to be in the field of vaccination or passive immunization for humans using chimeric (Murine-human) or genetically engineered MAbs. More than 10 million people annually receive rabies virus prophylaxis in the form of Equine rabies immunoglobulin (ERIG) or Human rabies immunoglobulin (HRIG), together with rabies vaccine (7). However, the world shortage of these immunoglobulins, as well as the risk of

both adverse reactions associated with ERIG and the high cost of HRIG, have hampered global efforts to provide PEP against rabies (23, 24). For these reasons, it is desirable to develop MAb to replace HRIG in the rabies PEP.

The present study was envisaged upon keeping these purposes in mind. In the present study, we aimed to produce anti-glycoprotein and anti-ribonucleoprotein MAbs. Also to determine the different reactivity patterns of fixed and street rabies virus strains with these MAbs. We intended to use indirect immunofluorescence technique. Antirabies MAbs can be used in diagnosis as well as to differentiate rabies strains. This study may possibly give us a diagnostic tool to identify rabies variants in limited time which will help to study the prevalence and transmission of antigenic variants within animal species in India.

REVIEW OF LITERATUR

HISTORY

"If a dog is mad and the authorities have brought the fact to the knowledge of its owner; if he does not keep it in, and it bites a man and causes his death then the owner shall pay two-thirds of a min (40 shekels) of silver. If it bites a slave and causes his death he shall pay 15 shekels of silver."

– Circa 23rd century BC

Based on the above description found on clay tablets in the Mesopotamian Laws of Eshnunna (Babylon), it can be seen that Rabies was known before 2300 BC. Ancient texts from Egypt, China, Persia, Palestine and India also appear to contain allusions to the disease. The first certain references to rabies are from Greek literature. The Greeks called rabies "Lyssa or Lytta which means madness. The disease in man was described as hydrophobia in which the sick person is tormented at the same time with thirst and the fear of water. The Latin word 'rabies' is derived from Sanskrit word '*rabhas*' which means 'to do violence.' Democritus has made the first recorded description of canine rabies around 500 BC. In fact, references to human rabies were found in the third century BC. The biological basis of the disease had started to become apparent from the nineteenth century. A strong scientific grasp on the transmission and progress of the disease has allowed many nations to initiate public health campaigns that have nearly wiped out the incidence of human rabies throughout the developed world (25).

By the end of the 19th century, an effective vaccination procedure was developed. Experimental transmission of rabies by inoculation of saliva was

first demonstrated in 1804 by German scientist Gottfried Zinke. Since the 1880's, there have been several significant advances in the diagnosis, treatment and laboratory study of rabies. In the beginning of 1881, Pasteur performed several experiments which showed that the disease was caused by a viral agent, and this agent multiplied primarily in the tissues of the central nervous system. This led to the development and testing of the first human rabies vaccine in 1885 (26). In 1921, this vaccine was adapted for use in domesticated dogs as part of a rabies control regime in Japan. In the 1940's the United States initiated programs for mass vaccination of dogs, and later for cats as well. Meanwhile in 1903, advances in rabies diagnosis have included the discovery of the Negri body, a characteristic dense matter seen via microscopy in the brain cells of about 75% of infected individuals. An improvement on this method came with the use of the more sensitive fluorescent antibody test for rabies diagnosis in 1959 (27). Another noteworthy achievement in the laboratory was the development of cell culture techniques for maintenance of rabies-infected cells, allowing investigators to characterize the virus and study its ability to infect (28). These medical and technological advances helped us to understand the pathology of rabies. This led to the dramatic decline in developed countries during the 1940's and 1950's.

In 1960's, Wistar Institute developed a rabies vaccine, which eliminated many problems associated with the brain tissue vaccine. Rabies virus grown in human diploid cells was used as a source for the production of safe and efficacious rabies vaccine by concentration and purification (29). This vaccine is now being used widely throughout the world. After that Purified Chick

Embryo Cell Culture Vaccine (PCECV), Purified Vero Cell Rabies Vaccine (PVRV) etc. were marketed. However, certain developing countries still use the brain tissue vaccine due to economical reasons. In 1954, the WHO trial on human subjects proved that the combined use of HRIG/ERIG and vaccine was much more protective than the vaccine alone (30). Currently, in cases of severe exposure HRIG/ERIG-vaccine treatment is recommended.

EPIDEMIOLOGY

Global scenario: Rabies is endemic world-wide, either in dogs or in wild animals, except in those limited areas of the world (mostly islands) where rabies has never existed or where it has been eliminated. Most developing countries of Africa, South and South-East Asia and to a lesser extent, Latin America have many cases of rabies in dogs which result in many human antirabies vaccinations and numerous human deaths. According to WHO, more than three billion people are at risk for rabies in over 85 countries and territories worldwide, and about 50,000–60,000 human deaths from rabies are estimated to occur annually, even though effective vaccines for PEP are available and over 10 million individuals actually receive rabies PEP each year (6,7). More than 99% of all human deaths from rabies occur in Asia, Africa and South America (7). The true disease burden of rabies is largely under-estimated, especially in Africa. For example, in Tanzania, incidence of human rabies mortality was estimated to be about 1,500 deaths per year (31). Serotypes 2, 3 and 4 have been isolated in Africa (31). Children under 15 years of age are the main victims of dog bites (30–60%) in canine rabies

endemic areas (31). In Central and Eastern Europe and North America, most human rabies cases are associated with contacts with wild carnivore species (e.g. foxes, raccoons, dogs, skunks and coyotes), which are the main reservoir of rabies virus, or with bats whose role has been considerably increasing in recent years (7). In Latin America there are two epidemiological cycles present with genotype 1. The first cycle is terrestrial, which is maintained by dogs and second is aerial which is carried by bats (32).

In the United States and Europe, rabies in domestic animals was largely controlled during the 1940–50s and now animal rabies record shows less than 10% cases (33). The following countries and regions are free of rabies: Scandinavia, Spain, Portugal, Greece, Japan, New Zealand, Guyana, Albania, Cyprus, Israel, Uruguay and Libya (34). Until recently this group included Australia and Great Britain; however the situation changed in 1996 when in both countries bats were diagnosed with rabies (5, 35). In Australia the infection was found in a frugivorous bat which belongs to serotype 7 in the *Lyssavirus* genus (36).

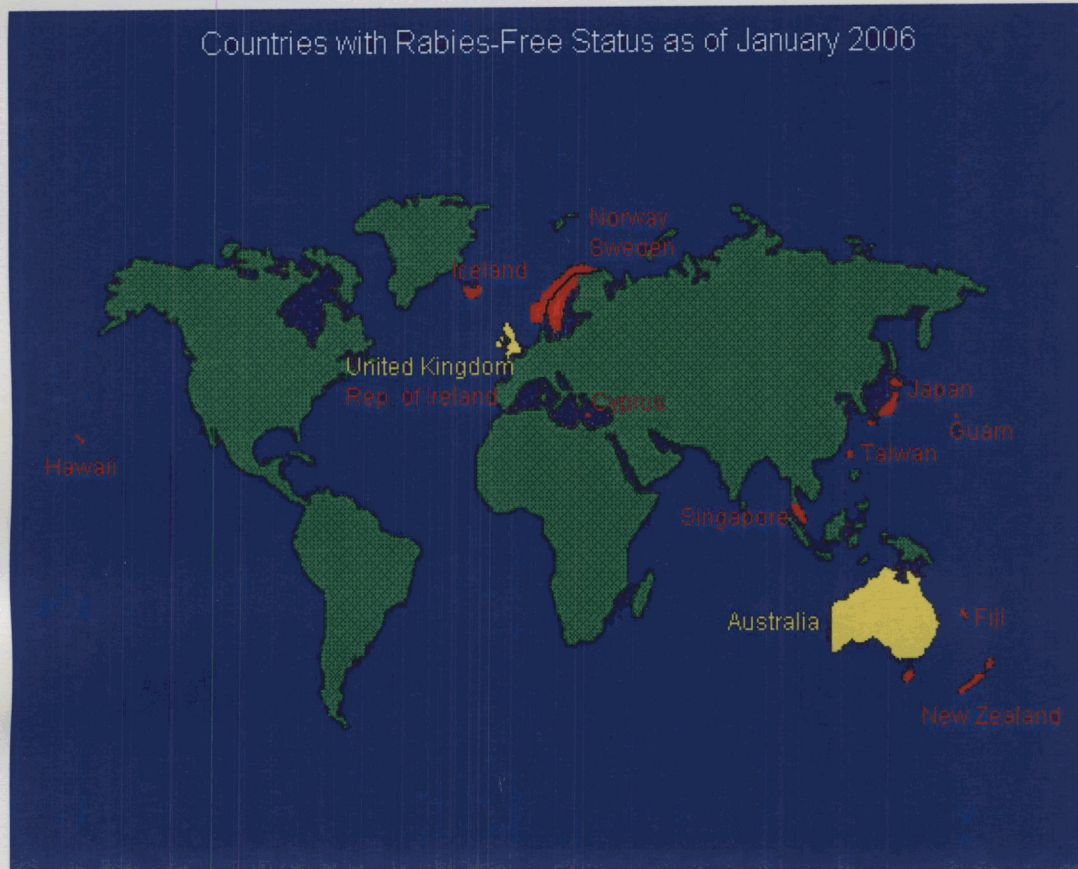
Asian scenario: In Asia, the main transmission route for rabies is a rabid dog bite. Between 94% and 98% of rabies deaths are due to canine bites (37). Rabies is particularly prevalent in over-populated countries (Bangladesh, India and Pakistan). Over the last few years, there has been a decrease in mortality rates in countries where efficient disease control measures have been implemented, e.g. Thailand and Vietnam (38). In other countries, such as the People's Republic of China and the Philippines, the number of cases has increased over recent years (5). Most people who die of rabies have not been treated or have not received adequate treatment because of the high cost of

rabies vaccines and immunoglobulins. Although WHO recommends to use only cell culture vaccines, some Asian countries (particularly Bangladesh and Pakistan) still manufacture cheap vaccines produced from infected brains. Australian Bat lyssavirus (ABLV) was found in insectivorous and frugivorous bats in Philippines (39). Over the last few years, four new isolates have been described in insectivorous bats. They are Arvana virus, Khujand virus, Irku virus and West Caucasian bat virus. All these viruses show similarity to rabies related viruses (5).

Indian scenario: In India, rabies is enzootic and is a serious public health and economic problem. Rabies is present uniformly in all states of India except in Andaman, Nicobar and Lakshadweep islands (40). In 2002, India alone reported 30,000 cases per year, i.e. an annual incidence of approximately 3 deaths per 1,00,000 population (7). It is unfortunate that in spite of availability of effective vaccines and sera, rabies takes such a heavy toll of lives in India. In this country, it is difficult to know the exact prevalence of the disease, therefore, usually it is assessed by the number of cases taking treatment after dog bite, by the deaths caused by hydrophobia and also by the confirmed positive cases of humans and animals in the laboratory (41). Survey conducted by the Association for Prevention and Control of Rabies in India (APCRI) has revealed startling facts: annually about 18,500 human deaths are due to rabies, out of which 89% had not taken post exposure vaccination, children constituting almost 50%, and 17 million animal bites annually out of which only half seek medical advice. This amply speaks of the level of public awareness about this dreaded disease. The annual number of patients receiving PEP in India is 1.07 million (8, 11, 42,). In India, rabies

occurs mainly in the urban form, although the existence of a sylvatic cycle can not be ruled out. In the urban form, dogs play an important role as the reservoir and transmitter of the disease to humans and domestic animals. India has a large population of stray dogs which, together with a lack of effective control strategies, might have led to the persistence of rabies virus. The human population considered to be at risk was calculated using the regional rural ratios for humans to dogs (43). The molecular epidemiology study by T. Nagarajan et al. shows the absence of a prevalence of rabies related viruses in India in domestic animals, including dogs (44).

Figure1. COUNTRIES WITH RABIES-FREE STATUS (IN RED), AS OF JANUARY 2006



- Countries where rabies is present
- Rabies free countries
- Rabies free in terrestrial animals



RABIES VIRUS

CLASSIFICATION

The rabies virus belongs to the Rhabdoviridae family, which consist of negative stranded RNA viruses. This family consists of six genera viz. *Vesiculovirus*, *Lyssavirus*, *Ephemerovirus*, *Novirhabdovirus*, *Cytorhabdovirus* and *Nucleorhabdovirus*. The rabies virus belongs to *Lyssavirus* genus, the order *Mononegavirales*. The virus was originally classified into 7 serotypes on the basis of serological and antigenic relationships: serotype 1 consists of 'classical' rabies viruses, notably the 'street' (wild) and vaccinal strains; serotypes 2, 3, 4, 5, 6 and 7 were rabies-related viruses prototyped by Lagos bat, Mokola, Duvenhage, European bat lyssaviruses (EBL1 and EBL2) and Australian bat lyssaviruses respectively. The vaccine prepared with serotype 1 may not show a protective effect against rabies related viruses (45). The distribution of rabies and rabies related viruses is shown in table 1.

**Table 1. VIRUSES OF THE FAMILY RHABDOVIRIDAE, GENUS
LYSSAVIRUS**

GENOTYPE	SEROTYPE	CROSS- REACTIVITY	DISTRIBUTION	SPECIES
Rabies	1	Lyssa 1	Worldwide except: Australia, UK, Ireland, New Zealand, Japan, Antarctica, Scandinavia, Hawaii	Domestic & wild carnivores, herbivores, bats
Lagos-bat	2	Lyssa 2	Africa	Fruit bats, cats, dogs
Mokola	3	Lyssa 2	Africa	Cats, dogs, rodents
Duvenhage	4	Lyssa 2	Africa	Insectivorous bats
European Bat Lyssavirus 1	5	Lyssa 1	Europe	Insectivorous bats
European Bat Lyssavirus 2	6	Lyssa 1	Europe	Insectivorous bats
Australian Bat Lyssavirus	7	Lyssa 1	Australia, Philippines	Fruit bats
Aravan virus	Proposed genotype	-	Southern Kyrgyzstan	Insectivorous bats
Khujand virus	Proposed genotype	-	Northern Tajikistan	Insectivorous bats
Irkut virus	Proposed genotype	-	Eastern Siberia	Insectivorous bats

Routes of infection:

Rabies infection in man is the result of a bite by an animal which has rabies virus in its saliva. Virus cannot penetrate unbroken skin but even a lick by an infected animal can be dangerous if the skin is damaged in some way. The virus can enter the body through the intact mucous membranes such as the conjunctival membrane covering the eye or the membrane lining the mouth, anus and external genital organs (46). Laboratory animals can be infected by eating food containing the virus. This route has never been confirmed in man. Inhaling airborne rabies virus into the nose and throat is the rarest recorded route of infection in man. The CDC states: "Inhalation of aerosolized rabies virus is also a potential non-bite route of exposure, but other than laboratory workers, most people are unlikely to encounter an aerosol of rabies virus". It has been suggested that people in infected bat caves may be exposed to aerosolized virus (46).

Post-vaccinal rabies is caused by injection of a vaccine which accidentally contains 'fixed' rabies virus which has not been properly inactivated. The saliva, tears, sputum and other body secretions of human rabies victims contain rabies virus. These patients may salivate, spit and cough over or even bite people near them. Also the virus can be transferred from an infected lactating mother to her child. In rare cases, rabies has been transmitted by corneal transplant or transplant of other tissues, or through contact of infected saliva with mucosal membranes or an open wound in the absence of a bite (35). Theoretically, any warm blooded animal could transmit rabies to man; however, more than 90% of cases result from bites by the

domestic dogs. Cats are second in importance, followed by other domestic and wild animals (47).

MORPHOLOGY AND STRUCTURE

Morphology

The virus particles have a bullet-shaped structure, one end is rounded or conical and the other end is planar or concave. The diameter of the virus is 75 nm and a length of 100–300 nm (15). Variations in the length can be observed between rabies strains, or can reflect the presence of defective interfering (DI) particles, which occurs when the multiplicity of infection is high. The lipoprotein envelope carries knob like spikes, composed of Glycoprotein G. Spikes do not cover the planar end of the virion. Beneath the envelope is the membrane or matrix (M) protein layer which may be invaginated at the planar end. The core of the virion consists of helically arranged ribonucleoprotein. The genome is unsegmented linear antisense RNA. The nucleocapsid consists of RNA dependent RNA transcriptase and some structural proteins.

Figure 2. ELECTRON MICROGRAPH OF NEGATIVELY STAINED RABIES VIRUS

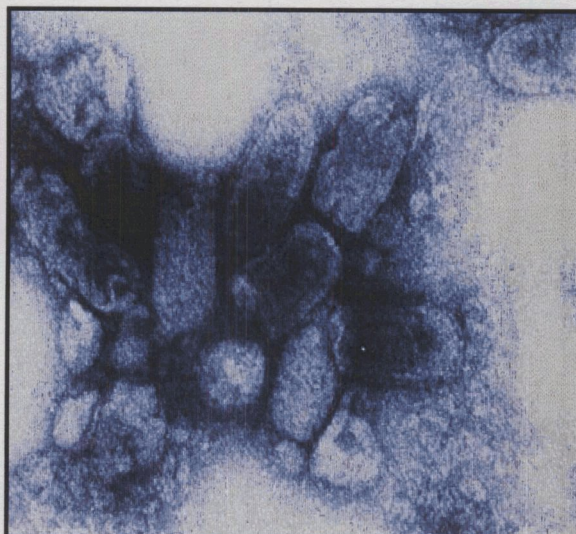
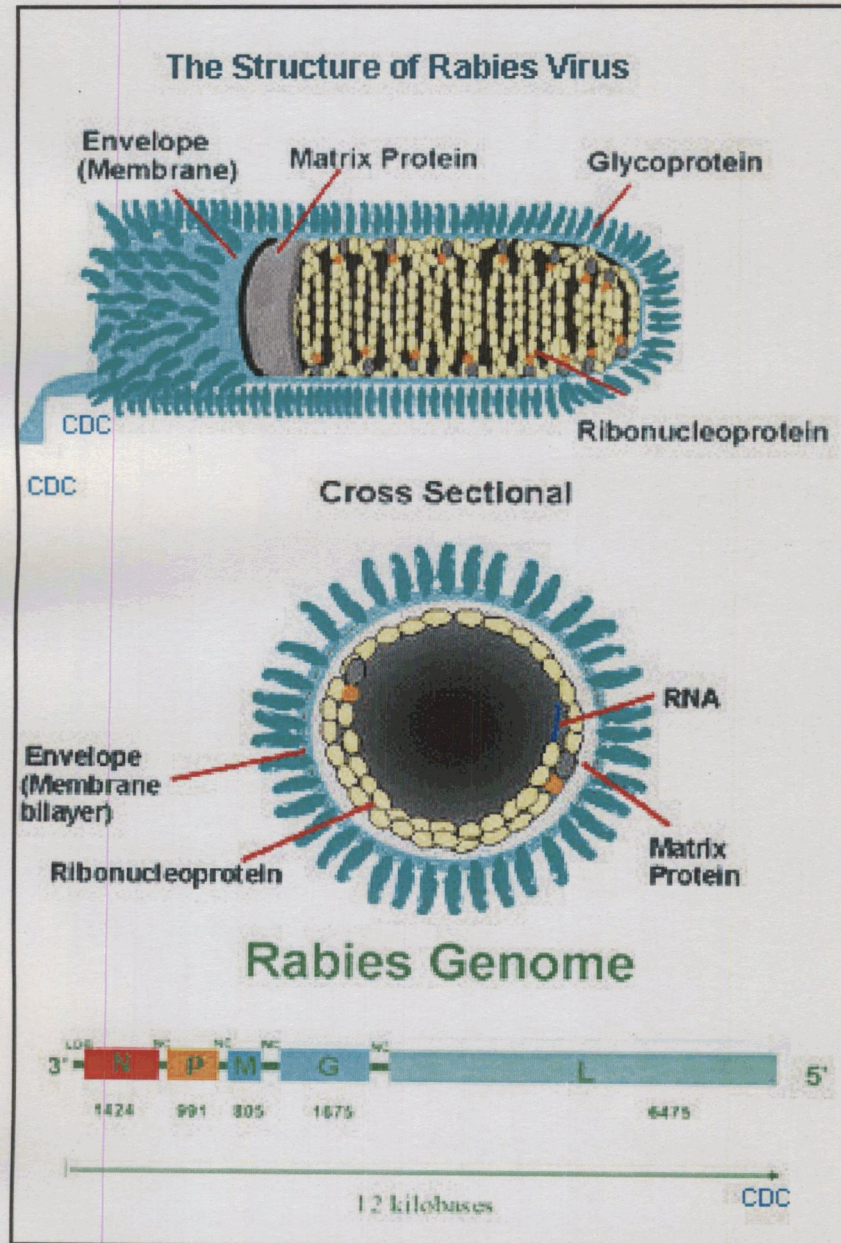


Figure 3. THE STRUCTURE OF RABIES VIRUS



Structural proteins

Biochemical studies have demonstrated that each virion is composed of a single molecule of genomic RNA and five proteins showing post-translational modifications (48):

1. The RNA-dependent RNA polymerase L protein (180 KD)

This is the giant protein of 2142 and 2127 amino acids in the PV and SAD B19 strains of rabies virus, respectively, occupies more than half (54%) of the rabies genome. L protein shows the sequence homology with other members of the order *Mononegavirales* (49).

2. Glycoprotein G exists in two forms G I (65 KD) and G II (70 KD)

The G protein is the best studied of the rabies proteins, at both the structural and immunological levels. It is 524 and 522 amino acids long in rabies and Mokola virus, respectively. The G protein is involved in the pathogenesis of rabies and is believed to assume at least part of the neurotropism of the virus. At least eight antigenic sites have been located on the external domain of the G protein of the different rabies strains (I-VI 'a' and G1) (50, 51, 52). The epitope site III of the rabies virus glycoprotein appears to be a major antigen responsible for protection. The proteolytic treatment of the virion removes the spike like projections and selectively affects the G protein. Only a small G protein remains linked with the spikeless particle indicating that the G protein is membrane anchored and constitutes the main component of the viral spikes. The spikes that contain the envelope glycoprotein are attached to the core particles probably with

the help of viral lipids. Each spike consists of three associated G proteins (trimers). There are about 1200 G proteins (400 trimers) per virus particle. There are three sugar chains that are N-glycosidically attached. Penetration of the virus into the cytoplasm takes place in the endocytic pathway and not at the plasma membrane. This is because the G protein trimer undergoes a change in conformation at pH 6.1, thus stabilizing the trimer and probably allowing the hydrophobic region of the molecule to become exposed and to get embedded in the membrane of the cell to be infected.

This virus G protein is responsible for induction of virus neutralizing antibodies and protection of animals against challenge, stimulation of specific T cells, which express helper, suppressor or cytotoxic activities, and determination of virulence. Because of its multiple immunogenic functions, this G protein is potentially suitable for vaccination purposes (50). Antigenic determinants carried on the G protein vary substantially among the rabies virus strains.

3. Nucleoprotein N (57 KD)

This is a 450 amino acid long polypeptide which is phosphorylated on a serine residue in position 389. One function of this protein is to encapsidate and protect the genome. Monoclonal antibodies directed against the N protein have been used to differentiate and classify rabies isolates into types of lyssaviruses (53).

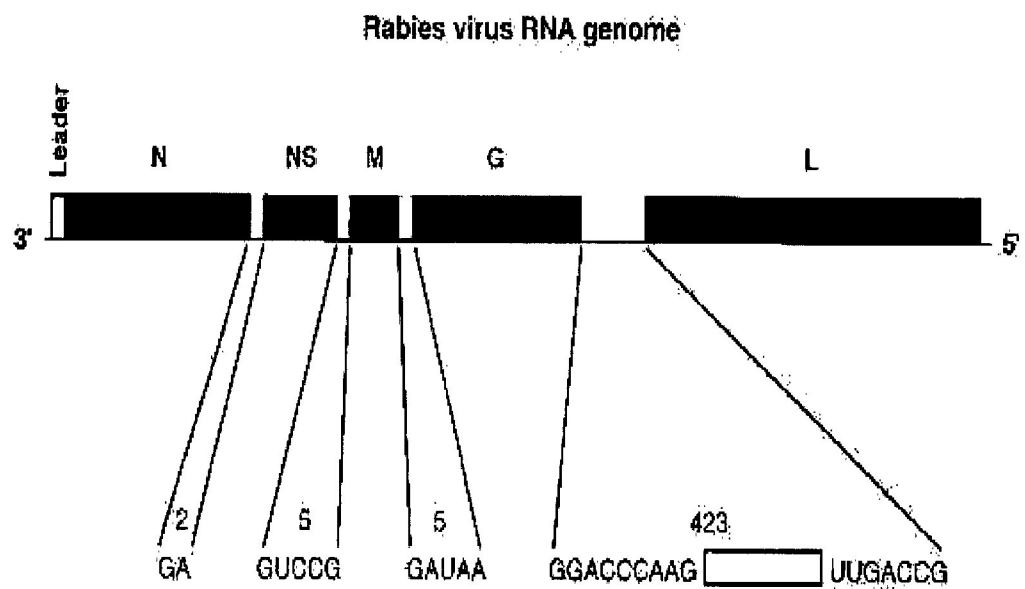
4. Phosphoprotein M1, exists in two forms M1 I (38 KD) and M1 II (41KD)

This is highly hydrophilic protein having 303 amino acids. In the year 1981 it was suggested that M1 protein is not a membrane protein but is found to be occupying an internal position within the virion in association with N protein (54).

5. Matrix or membrane protein M2, exists in two conformation: M2 I (24 KD) and M2 II (25 KD)

This is a 202 amino acid long polypeptide. It plays an intermediate role between the ribonucleocapsid and the viral membrane, presupposing the presence of regions interacting with both elements. It is believed to play an important role in morphogenesis of the virus. The association of two membrane proteins with the nucleocapsid results in folding of the M2 protein and its enclosure by membrane.

Figure 4. RABIES VIRUS GENOME



REPLICATION OF RABIES VIRUS

Adsorption

It is widely accepted that many viruses are taken into cells by a process called phagocytosis. In the case of enveloped viruses, fusion may occur between the envelope and the cell membrane. Both processes have been observed in cultures of baby hamster kidney (BHK-21) cells infected with rabies virus. The binding of the rabies virus to the cell membrane is thought to be mediated by the G protein. On the basis of sequence homology between the external part of the G protein and the receptor binding site of snake venom neurotoxins, it was postulated that the rabies virus could bind to the nicotinic acetylcholine receptor (55). In neuronal and fibroblastic cells, it has been shown that oligosaccharides and lipoprotein components such as sialic acids and gangliosides may also be involved (15, 56).

Penetration and Uncoating

Once fixed on the cell, the rabies virion penetrates it by pinocytosis. The viral membrane then fuses with that of the lysosomal vacuole, releasing the ribonucleocapsid into the cytoplasm. The ribonucleocapsid is sufficient to assure the transcription of the virus.

Transcription, replication and budding

The rabies virus possesses an unsegmented negative-stranded RNA genome. The negative polarity means that the genome is unable to be directly translated into viral proteins by the cell machinery. Therefore, a preliminary autonomous transcription step is necessary to produce the complementary

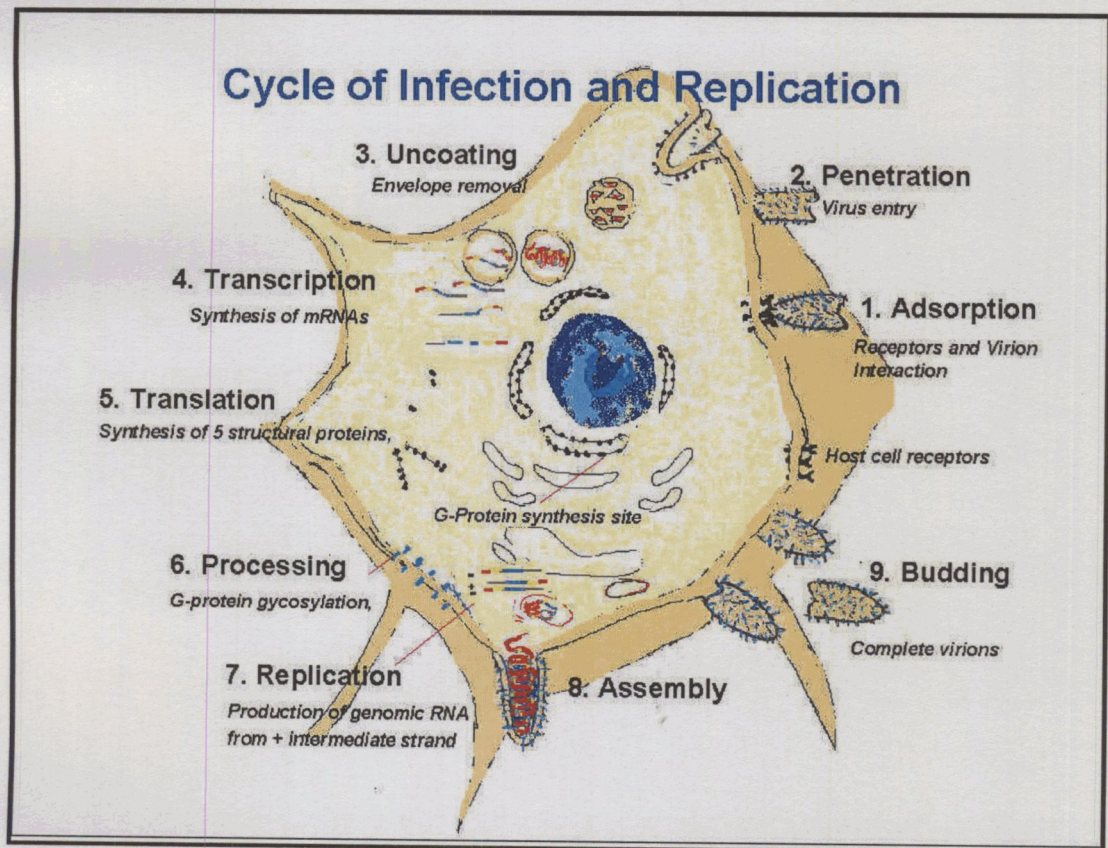
positive-stranded messenger RNA (mRNA), as soon as the ribonucleocapsid is liberated in the cytoplasm. This step is assured by a genome-encoded enzyme, the RNA-dependent RNA polymerase, which is included in the ribonucleocapsid. The viral polymerase does not recognize a naked RNA template (15).

A viral-encoded polymerase (L gene) transcribes the genomic strand of rabies RNA into leader RNA and five capped and polyadenylated mRNAs, which are translated into proteins. Translation, which involves the synthesis of the N, P, M, G and L proteins, occurs on free ribosomes in the cytoplasm. Although G protein synthesis is initiated on free ribosomes, completion of synthesis and glycosylation (processing of the G protein) occurs in the endoplasmic reticulum (ER) and Golgi apparatus. The intracellular ratio of leader RNA to N protein regulates the switch from transcription to replication. When this switch is activated, replication of the viral genome begins. The first step in viral replication is synthesis of full-length copies (positive strands) of the viral genome. When the switch to replication occurs, RNA transcription becomes "non-stop" and stop codons are ignored. The viral polymerase enters a single site on the 3' end of the genome, and proceeds to synthesize full-length copies of the genome. These positive strands of rabies RNA serve as templates for synthesis of full-length negative strands of the viral genome.

During the assembly process, the N-P-L complex encapsulates negative-stranded genomic RNA to form the RNP core, and the M protein forms a capsule, or matrix, around the RNP. The RNP-M complex migrates to an area of the plasma membrane containing glycoprotein inserts, and the M-protein initiates coiling. The M-RNP complex binds with the glycoprotein, and the

completed virus buds from the plasma membrane. Within the central nervous system (CNS), there is preferential viral budding from plasma membranes. on the other hand, virus in the salivary glands buds primarily from the cell membrane into the acinar lumen. Viral budding into the salivary gland and virus-induced aggressive biting-behavior in the host animal maximize chances of viral infection of a new host (57).

Figure 5. REPLICATION OF RABIES VIRUS



RABIES PATHOGENESIS

Rabies viruses differ in their pathogenicity. Certain laboratory adapted viruses (i.e. fixed viruses) are highly invasive when injected either intracerebrally or intramuscularly. Laboratory strains of 'fixed' virus used to produce vaccines are employed in diagnostic and research procedures. The pathogenicity also depends on the species of animal bitten.

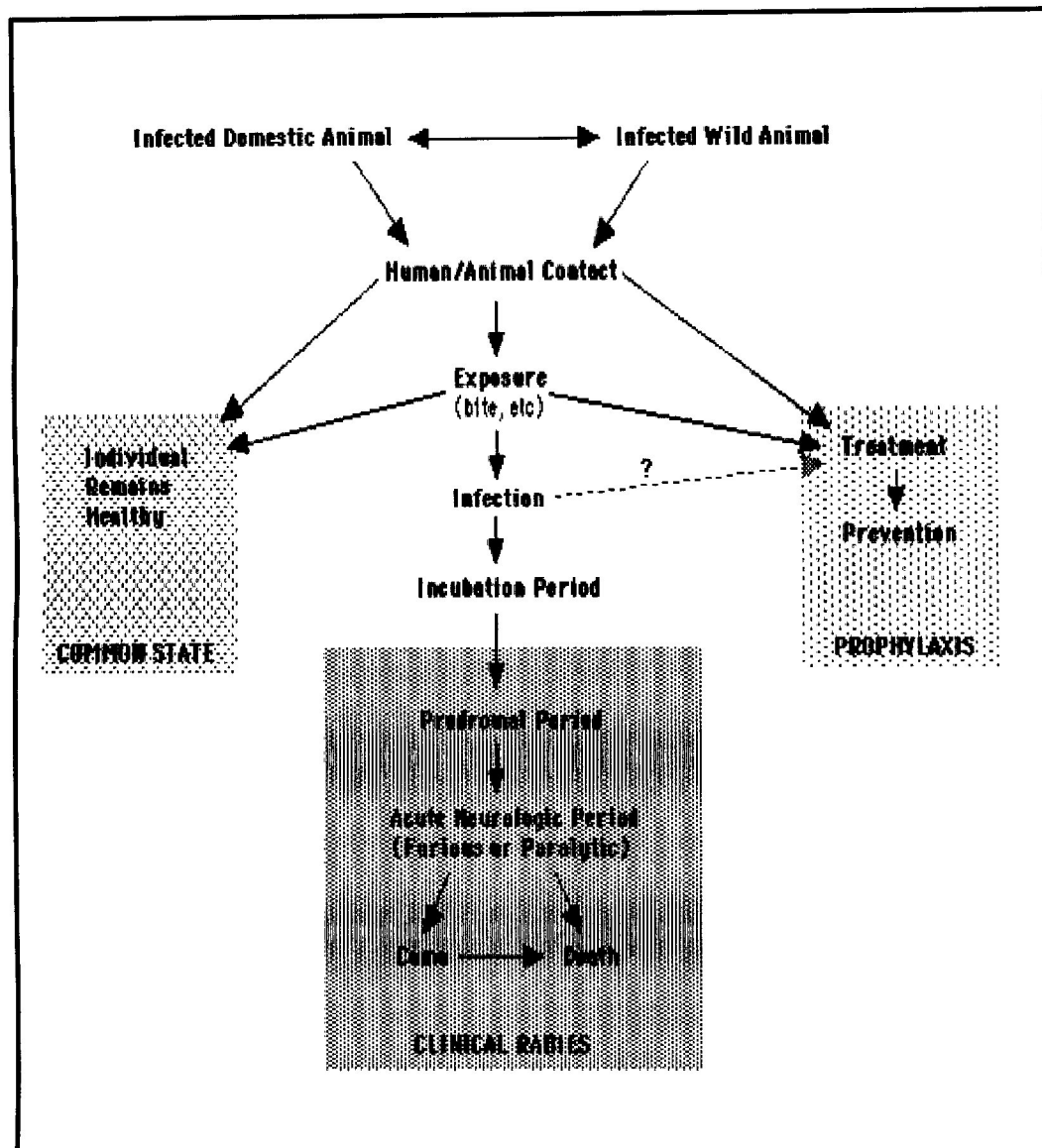
The virus is usually introduced by a bite wound, although penetration can occur through intact mucous membranes and the digestive tract, but not through intact skin (58). The virus replicates at the site of inoculation and then enters a peripheral nerve. It then travels along the nerve (retrograde axoplasmic flow) to the dorsal root ganglia. It replicates here and gains entry to the central nervous system and spreads to various parts of the brain. From here it can spread out via the nerves to various sites in the body. The antibody response in an unvaccinated person is not seen until after the disease develops. If there is disease, it almost inevitably results in death.

Statistics on human disease also indicate that wounds on the head and neck are generally more lethal than those of hand and feet (59). After the short initial period of obvious viability the virus goes into an eclipse phase, where it cannot be recovered from any tissue. During this period the virus may have remained locally or already proceeded on its pathway to or within the CNS. There is also evidence that virus stays locally for a long time at the site of wound or inoculation (60).

In 1973, Murphy and colleagues elucidated the pathogenesis of rabies infection in an exquisite series of studies (61). They reported that rabies infections are generally established by virus inoculation into peripheral tissues and ascend via afferent peripheral nerves and reach brain neurons. Although the usual method of transmission is inoculation of infected saliva into subcutaneous tissue, muscle or onto broken skin, the other modes of transmission have also been recognized viz. virus inhalation, transplantation of corneal tissue containing virus and inoculation onto mucous membranes. On inoculation of the virus into the striated muscle cells, the virus begins to replicate. Electron microscopy studies on myocytes revealed virus particles budding from plasma and sarcoplasmic reticulum membranes of infected cells and amplifying the number of virions locally. This remarkable finding that striated muscle probably is the site of virus sequestration in long incubation infection has a great importance in the study of PEP. This is due to the fact that the efficacies of treatment procedures most probably depend on the accessibility of virus before it becomes ensconced in the nervous system. Within 62–72 hours of inoculation in dorsal root ganglion cells the virus ascends into spinal cord. Rapid infection of spinal cord, brain stem, cerebellum and other brain structures follows. The virus then descends via sensory and autonomous nervous system to retina, cornea, salivary glands, skin, nasal mucosa and other organs and tissues subsequently. Besides the direct viral replication in the salivary glands, the high concentration of virus in saliva results from viral shedding from sensory nerve endings in the oral mucosa (62)

Natural rabies infection may require a period of local viral replication, presumably to increase the concentration of inoculum, before nervous system infection can occur. During this period, the administration of antirabies immunoglobulins and the development of (antirabies) immunity by active immunization are able to prevent spread of the virus into the nervous system and are thereby able to prevent clinical disease. Once the virus enters the peripheral nerve, current therapeutic techniques may not be able to prevent subsequent replication of the virus. However, advanced research techniques hold the promise of antibody delivery across the blood brain barrier, which in turn may allow more effective treatment (63).

Figure 6. COURSE OF RABIES TRANSMISSION AND PROGRESSION



IMMUNE RESPONSES

Humoral Immune Responses

Humoral immunity clearly provides protection against the infection. Because the G protein spikes of rabies virus represent the only antigen that induces virus-neutralizing antibodies (VNAs) and also be able to confer immunity against lethal challenge infections (64, 65). The intact secondary and tertiary structure of spike G protein is important to induce VNAs and also to confer host immunity (66). The mechanisms whereby VNAs exert their effect include: a) Neutralization of extracellular rabies virus b) Complement mediated lysis of virus infected cells and c) Antibody dependent cellular cytotoxicity (67). The RNP of rabies virus is also a major antigen that induces a virus specific antibody response, and antibodies directed against RNP also may contribute to protection against rabies virus infection (68). In absence of vaccination, antibodies in naturally acquired infection occur only after well establishment of the disease. Serum neutralizing antibodies generally do not appear until the 5th day of the onset of rabies symptoms (69). They may be also detected in cerebrospinal fluid (CSF).

Cellular Immune Responses

Hale (1978), reported the rabies virus infection results in the generation of virus specific CD8⁺ and CD4⁺ T cells. The viral G protein appears to be the major antigen that mediates a cytotoxic T cell response and also is its major target (70). In animals, the live rabies virus develops a strong CTL (Cytotoxic T- Lymphocytes) response specific to NS protein. The rabies nucleocapsid is

also a major antigen that activates CD4⁺ cells, and the majority of these T cells cross-react among different Lyssaviruses. The RNP specific T cells help in production of VNA by intracellular antigen recognition and mediate the protective immune response induced by RNP. The immune mediated cytolysis of rabies infected cells results in death of neuronal cells, detrimental to host. Rabies infection usually induces the production of interferon, which inhibits infection in tissue culture (71). And administration of exogenous interferon or interferon inducers demonstrated their ability to protect against rabies infection. The production of interferons in natural infection is not sufficient enough to protect an unvaccinated individual. Delayed processing and response by the immune system is due to the small amount of viral antigen early in the infection and also the intracellular location.

CLINICAL DESCRIPTION

Clinical rabies in man can be discussed by following five stages: incubation period, prodrome, acute neurologic phase, coma and death or rare recovery (72).

1. **Incubation period:** The reported incubation periods for rabies ranges from as short as 5 to 6 days, but, in the majority of cases, the incubation period is between 20 and 60 days (73). Although incubation periods of longer than 6 months are reported in less than 1% cases. Incubation periods are usually shorter when the site of the bite is on the head rather than an extremity and is shorter in children than adults. Without vaccination, the risk of death varies with the quantity of virus in the saliva of the biting animal, and the severity and location of the bite. During the incubation period, there are no symptoms and no means of diagnosis; clinical illness begins with nonspecific prodrome- like symptoms, slight fever, headache, malaise, anxiety lasting for few hours to few days (72).
2. **Prodrome:** the first symptoms of clinical rabies are usually malaise, anorexia, fatigue, headache and fever. In about half of the individuals pain or paresthesias are reported at the site of the exposure. Apprehension, anxiety, agitation, irritability, nervousness, insomnia or depression may be prominent during this period.
3. **Acute neurologic phase:** Two to 10 days after onset of prodromal symptoms the patient develops objective signs of nervous system involvement usually including hyperactivity, disorientation, hallucinations,

seizures, bizarre behaviour, paralysis. As in animals, a period of hyperactivity lasting hours to days develops in a majority of cases. In humans, this hyperactivity is characteristically intermittent and consists of periods of agitation lasting 1 to 5 minutes. Hydrophobia (the fear of water) appears to develop in most cases of furious human rabies. Attempts to drink or eat may produce severe painful spasms of the pharynx and larynx producing choking, gagging and fear. Other abnormalities during this phase include muscle fasciculation, hyperventilation, hypersalivation, convulsions. Paralysis may be symmetric, asymmetric with maximal involvement in the bitten extremity or ascend as in the Guillain-Barre syndrome (69). During this phase the mental status gradually deteriorates from confusion to disorientation, stupor and finally coma.

4. **Coma:** The period of coma may last for hours or months, depending on the intensity of care. Without supportive care, respiratory arrest usually develops shortly after the onset of coma and leads to death, within 5 days for furious rabies and 13 days for paralytic rabies (74).
5. **Death or rare Recovery:** Without PEP the disease is 100% fatal, recovery is very rare after the onset of acute neurologic phase. To date, there are fewer than six cases of known survival among humans who showed signs of disease, all of whom recovered with sequelae and one with severe psychogenic disturbance (42, 75, 76). All survivors received either pre-exposure or post-exposure rabies vaccination.

TREATMENT

LOCAL WOUND TREATMENT

All wounds should be washed immediately and thoroughly for several minutes with soap and water. This is the single most procedure in preventing infection. After washing the chemical disinfectant (Quaternary ammonium compounds or iodine solutions) should be applied. Suturing of wounds should be delayed for as long as possible. Local infiltration of wounds with human or antirabies immunoglobulin (HRIG) should also be employed (77).

RABIES VACCINE AND IMMUNOGLOBULIN

There is no treatment for rabies after symptoms of the disease appear. However, two decades ago scientists developed an extremely effective new rabies vaccine regimen that provides immunity to rabies when administered after an exposure (post-exposure prophylaxis) or for protection before an exposure occurs (pre-exposure prophylaxis). Tissue culture Human Diploid Cell Culture Vaccine (HDCV), Purified Chick Embryo Cell Culture Vaccine (PCECV), Purified Vero Cell Rabies Vaccine (PVRV) etc. are some of the examples. These vaccines are preferred because of demonstrable safety and efficacy. But, Semple vaccine, Suckling Mouse Brain Vaccine and Duck Embryo Vaccines are discontinued in most of the developed countries due to their adverse side reactions (78).

Pre-exposure Prophylaxis

Pre-exposure vaccination may be performed with any of the modern cell-derived vaccines and is recommended for persons in high-risk groups, such as veterinarians, animal handlers, and certain laboratory workers. Other persons whose activities bring them into frequent contact with rabies virus or potentially rabid bats, raccoons, skunks, cats, dogs, or other species at risk of having rabies should also be considered for pre-exposure prophylaxis. In addition, international travellers likely to come in contact with animals in areas of enzootic dog rabies which lack immediate access to appropriate medical care, including biologics, should be considered for pre-exposure prophylaxis. People who work with live rabies virus in research laboratories or vaccine production facilities are at the highest risk of inapparent exposures. Such persons should have a serum (blood) sample tested for antibody every 6 months and receive booster vaccine, when necessary. Routine pre-exposure prophylaxis for other situations may generally not be indicated (79).

Pre-exposure prophylaxis consists of three doses of rabies vaccine given on days 0, 7, and 21 or 28. Depending on the vaccine type, a dose of 1 ml or 0.5 ml is administered intramuscularly. Major vaccine manufacturers recommend 1 booster dose after 1 year, and to ensure protection in persons at continued risk (78).

Post-exposure prophylaxis

Post-exposure prophylaxis (PEP) is indicated for persons possibly exposed to a rabid animal. Possible exposures include animal bites, or mucous membrane contamination with infectious tissue, such as saliva. Administration of rabies PEP is a medical urgency, not a medical emergency. Physicians should evaluate each possible exposure to rabies and as necessary consult with local or state public health officials regarding the need for rabies prophylaxis. In some cases, vaccination with rabies vaccine alone may be sufficient, whereas in others additional prophylaxis with rabies immune globulin (RIG) may also be required (80). The indication of post exposure vaccination with or without RIG depends on the type of contact with the suspected animal. According to WHO, types of contact are: category I- touching or feeding animals, licks on the skin; category II- nibbling of uncovered skin, minor scratches without bleeding, licks on broken skin; category III- single or multiple transdermal bites or scratches, contamination of mucous membrane with saliva from licks. For category I no treatment is required, whereas for category II immediate vaccination and for category III immediate vaccination and administration of rabies immune globulin are recommended in addition to immediate washing of all bite wounds and scratches (7, 82).

Depending on vaccine type, the post exposure schedule prescribes intramuscular doses of 1 ml or 0.5 ml given as 4–5 doses. The PEP regime recommended in the United States and by the WHO is rabies immune globulin (RIG) on day 0 and a cell culture vaccine on each of days 0, 3, 7, 14 and 28 (78, 82).

If post exposure treatment is given to immunocompromised individuals, HIV-positive persons, people under malaria chemoprophylaxis, intramuscular vaccine and RIG are mandatory and their antibody responses should be monitored serologically.

RABIES IMMUNOGLOBULINS (RIG)

Rabies immunoglobulins should be used along with the tissue culture vaccines for PEP following exposure to a suspected or proven rabid animal to provide protection against rabies. The WHO recommends the use of RIG for all category III exposures and for category II exposures (82). Both purified equine rabies immunoglobulin (ERIG) and human immunoglobulin (HRIG) are used in developing countries. The infiltration of RIG directly into and around the wound site(s) provides an exposed patient with an immediate source of rabies virus neutralizing antibodies (VNA). These VNA bind to and neutralize rabies virus that may have been inoculated into the wound at the time of exposure, thus preventing virus entry into the peripheral nervous system. This immediate, short-term protection is essential because it takes approximately 7–10 days for the humoral immune system to produce a measurable level of VNA after rabies vaccine has been administered. A skin test must be performed prior to the administration of ERIG. As much as possible of the recommended dose (20 IU/Kg of body weight of HRIG or 40 IU/Kg of body weight of ERIG) should be infiltrated around the wounds (79). Rabies immunoglobulins should be administered at the same time as the first dose of rabies vaccine. Rabies immunoglobulins may be diluted 2–3 fold with sterile saline to ensure sufficient volume to infiltrate multiple wound sites. Human

rabies immunoglobulin is available in some countries, however it is expensive and only limited amounts are available. Equine rabies immunoglobulin is available in many countries and is considerably cheaper than HRIG. Most of the currently available preparations of ERIG are highly purified and quite safe. Equine rabies immunoglobulin preparations from various manufacturers also showed serum sickness in some recipients (77).

TREATMENT FAILURES

Thraenhart and colleagues reviewed 28 cases of rabies that developed despite post-exposure treatment with modern vaccines (83). In 90% cases, RIG had not been administered or had been administered incorrectly. Other errors included passive immunization more than 2 hours before vaccine, incorrect local wound cleansing, injection of vaccine into the buttocks instead of the deltoid and late initiation of immunization. In case of immunosuppressed persons, HIV infected patients, patients on immunosuppressive drugs and those with diabetes may not respond to rabies vaccines (84, 85, 86). Apart from these reasons, the antigenic differences of rabies viral proteins are responsible for vaccine failures in some cases. The antigenic difference between the vaccine strain and street strains and/or rabies related viruses may lead to vaccine failure. A follow-up study of treatment failures after 15 million doses of purified chick embryo cell culture vaccine reported 47 treatment failures. All had occurred in India and Thailand (87)

DIAGNOSIS

The diagnosis of rabies is not difficult if a history of exposure is present and the full spectrum of symptoms and signs has appeared. Laboratory diagnosis may not be possible during the first week of illness. Clinical diagnosis of rabies requires differentiation from a wide variety of diseases that can cause neurologic symptoms. Rapid and accurate laboratory diagnosis of rabies in humans and other animals are essential for timely administration of PEP. In addition, laboratory identification of positive rabies cases may aid in defining current epidemiologic patterns of disease and provide appropriate information for the development of rabies control programs (88, 89).

Animal and human rabies diagnosis can be made by four methods: i) Histopathology ii) Virus cultivation iii) Serology and iv) Virus antigen detection.

Antemortem diagnosis:

Once clinical symptoms are present, there are several antemortem assays that can be performed to confirm the diagnosis of rabies. The laboratory specimens that should be examined include saliva, serum, spinal fluid, tissue biopsies and corneal smears. Laboratory tests include virus isolation, Reverse transcription polymerase chain reaction (RT-PCR), Immunofluorescence test and virus neutralization assay.

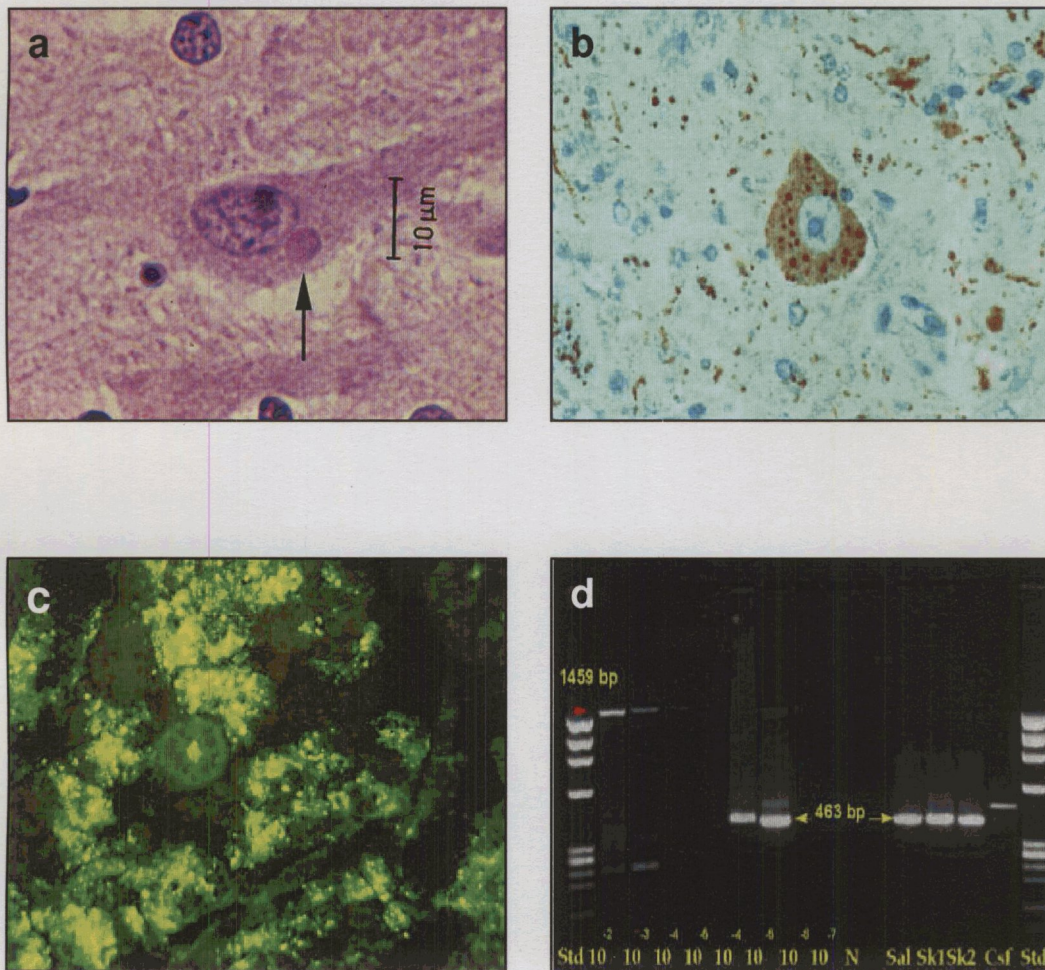
Virus isolation is possible from infected tissues using human diploid cell line (WI-38), baby hamster kidney cell line (BHK-21) and chick embryo related cells (CER) cell lines, since rabies virus induces a cytopathic effect (CPE). Presence of rabies antigen as well as antibodies can be detected by Enzyme

Linked Immunosorbant Assay (ELISA) (90). Rabies infection or vaccination usually results in antibody production, which can be measured by Mouse Neutralization Test (MNT) (91) or Rapid Fluorescent Focus Inhibition Test (RFFIT) (92). Additionally recent technological advances have facilitated the detection of viral RNA in urine samples from patients exhibiting rabies using nucleic acid sequence-based amplification (NASBA) (88, 93, 94).

Postmortem diagnosis:

Postmortem samples include Brain tissues, salivary glands. Definitive diagnosis of rabies infection of humans and suspected animals depends on the detection and identification of infected brain tissue of rabies virus antigen or intracytoplasmic inclusion bodies (Negri bodies) by Sellers staining and Histopathology. The standard test for identifying rabies over past 40 years has been the direct fluorescent antibody (DFA) test for identification of rabies virus in the tissue(s) examined (95). Monoclonal antibodies against rabies and rabies related viruses are used to detect and differentiate rabies strains (53, 96, 97). Other procedures include electron microscopy, the indirect fluorescent antibody (IFA) test, *in vitro* and *in vivo* virus isolation. Recent technological advances such as molecular hybridization and PCR provide definitive evidence of rabies infection (98).

Figure 7. TECHNIQUES IN LABORATORY DIAGNOSIS OF RABIES



- a.** Negri body in rabies infected neuron in Sellers stained brain tissue.
- b.** A rabies virus-infected neuronal cell with intracytoplasmic inclusions by using immunohistochemistry technique.
- c.** A frozen section of submaxillary salivary gland of rabies infected raccoon by direct immunofluorescence technique (300X magnification).
- d.** PCR test results for rabies virus. The arrows indicate positions of positive bands.

MONOCLONAL ANTIBODIES (MAbs) AS A DIAGNOSTIC TOOL

The conventional polyclonal antisera contain a heterogeneous population of antibodies. These vary from animal to animal and batch to batch in the same animal. The sera also contain undesirable non-specific or cross-reacting antibodies and removal of these unwanted antibodies is a time consuming task involving repeated adsorption techniques. The specificities and avidity of these antisera show a lot of variation. The hybridoma technique overcomes most of the above difficulties. An impure antigen can be used to generate very pure antibodies. The MAbs are homogeneous with a single specificity and affinity. Once a stable clone producing a particular MAb is established, it provides a continuous and unlimited supply of highly specific and pure antibodies (99).

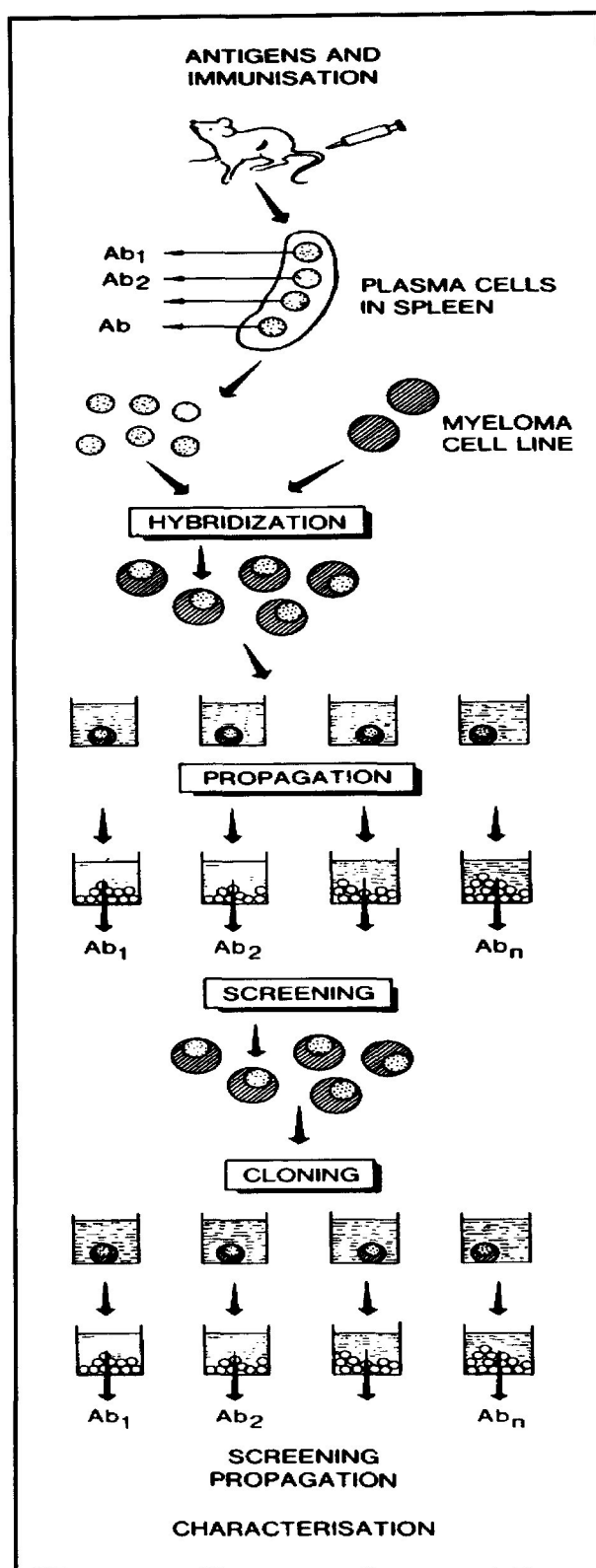
In 1975 Kohler and Milstein reported successful production of MAbs using hybridoma technique (21). Nearly 25 years after this discovery, MAbs continue to evolve from remarkably useful laboratory reagents into potent tools for the diagnosis and treatment of various diseases. A monoclonal antibody is produced by an immortal cell line derived from the fusion of a malignant Myeloma cell with a B lymphocyte, usually derived from the spleen of an immunized mouse. The Myeloma line is deficient in one of the essential salvage pathways for DNA synthesis and so does not survive when the main biosynthetic pathway for DNA synthesis is blocked. The unfused spleen derived lymphocytes do not survive because they are physiologically programmed to die unless 'rescued'. The only cells which survive in the appropriate medium are hybridomas, the Myeloma-spleen cell hybrids.

resulting from the fusion. The hybridoma derives its immortality and antibody secreting capacity from the Myeloma cell genes, and the salvage pathway genes and antibody coding genes from the spleen B lymphocyte (100, 101). The resulting clones of hybridoma cells, which secrete large quantities of MAbs, can be cultured indefinitely.

The development of hybridoma technology gave immunologists a powerful and versatile research tool. Monoclonal antibodies are proving to be very useful as diagnostic, imaging and therapeutic reagents in clinical medicine. Initially MAbs were used primarily as in vitro diagnostic reagents. Now these are used to diagnose many pathogenic microorganisms and viruses, measuring the blood levels of various drugs, matching histocompatibility antigens and detection of certain tumor antigens by radiolabelled MAbs (101). Antibody based specific detection, identification and differentiation of antigens can be done by immunostaining, immunoprecipitation, immunoblotting and immunofluorescence reactions. In the present study, MAbs plays an important role in diagnosis of rabies virus antigens.

The different stages in the production of MAbs, starting from immunization and finishing with a characterized MAbs are illustrated schematically in Figure 6. Each step is discussed in detail in Materials and Methods.

Figure 8. SCHEMATIC REPRESENTATION OF HYBRIDOMA
TECHNOLOGY



Rabies epidemiological studies presented a major advance since the 80's, after the availability of MAbs in antigenic analysis. Wiktor and Koprowski (1978) produced MAbs through the immunization of BALB/c mice by the inactivated rabies vaccine (96). The use of these MAbs in Indirect immunofluorescence technique has greatly contributed to epidemiological studies. Monoclonal antibodies against the various antigenic determinants of the nucleocapsid and glycoprotein of rabies virus have been prepared in several WHO Collaborating Centres. These antibodies can differentiate between the different rabies virus types. In particular, they can be used to identify wild strains of rabies virus originating from different geographical locations and reservoir species, as well as fixed strains used for rabies vaccine production. Limited panels of MAbs and reference reagents are available from WHO Collaborating centers for specific research projects, or the strains to be characterized may be submitted to one of these centers for identification.

In America and the Caribbean, several researchers conducted epidemiological studies using MAbs (102, 103). Antigenic and immunogenic studies using MAbs with rabies virus strains isolated in Brazil were carried out by Germano et al. and Hayashi et al. (104). In 1996, Pasteur institute of Sio Paulo implemented an indirect immunofluorescence technique, using a panel of MAbs against viral nucleoprotein, produced by the Centers for Disease control and prevention (CDC). They studied the antigenic typing of sample isolated in the America (105). Favoretto, Regina S. studied the antigenic variants from the samples isolated between 1989 and 2000 in different Brazilian regions from several species (106). Although, the MAbs are mainly

used for epidemiological investigations, they are also used for rabies diagnosis in circumstances of uncertain exposure (107, 108) and also routinely in countries where large-scale programmes for oral vaccination of foxes. MAbs have also been used to locate antigenic sites on the rabies virus N protein (109). MAbs has a very important application to identify functional antigenic map of rabies Glycoprotein (110).

Candidate vaccines have been prepared using anti-idiotypic MAbs directed against antigenic determinants in the rabies protein (111). Possible replacement of HRIG or ERIG by MAbs has been investigated in hamsters and mice treated after infection with field strains of rabies and other Lyssaviruses; preliminary results appeared promising (112, 113). The greater role for rabies MAbs may prove to be in the field of vaccination or PEP for humans using chimeric (Murine-human) or genetically engineered MAbs. Prosnia M et al. used DNA recombinant technology to express three rabies virus neutralizing human MAbs in a rhabdovirus vector. These recombinant expressed MAbs were used for the protection of rabies infected mice and hamsters. The study reveals the possible role of recombinant human MAbs for PEP of humans in future (114). Thus anti-rabies MAbs would be a best tool for diagnosis as well as for PEP in rabies infections.

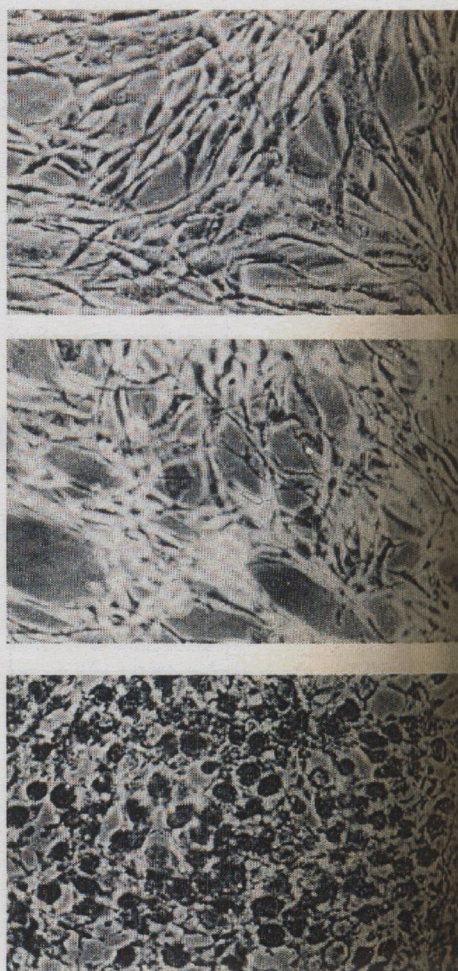
CELL CULTURE OF RABIES VIRUS

The advent of cell culture and the successful propagation of rabies virus in non-nervous mammalian cells had made a systematized approach to the study of rabies virus and the large scale production of the virus. The tissue culture technique to the study of rabies virus was first applied by Noguchi and Levaditi in 1913 (115). Atanasiu et al. cultivated street and fixed strains of virus in non-neural cells of a mouse ependymoma tumor cell line (116). The susceptibility of a variety of primary kidney cell cultures (mouse, hamster, pig, dog and monkey origin) and avian embryo fibroblasts to rabies virus demonstrated that these cells could be used for rabies vaccine production.

There are number of continuous cell lines which are used for production of rabies vaccines for animals such as baby hamster kidney cell line 21 (BHK-21), hamster kidney fibroblasts (Nil-2) and chick embryo related cells (CER) (117, 118). Because of the heteroploid characteristics and carcinogenic potential of these cell lines, only Vero cells have been considered suitable for the production of human vaccines (119). The production of first vaccine in human diploid cell line (WI-38) was first described in 1964. Neuroblastoma cells of human or murine origin are now widely used in rabies virus investigations. There is no clearly definable cytopathic effect accompanies the production of rabies virus in cell cultures. In infected BHK-21 cell monolayers the cells merely begin to age and detach more quickly from the supporting surface than uninfected control cells (120). Similarly, plaques were formed in a sub-line (S13) of BHK-21 cells adapted to growth in agarose suspension. This led to the development of the first reliable plaque assay system of rabies

virus. Plaque formation was also seen in infected Vero cells. Murine Neuroblastoma cells infected with rabies and rabies related viruses shows more severe cytopathic effect than BHK-21 cells.

Figure 9. Rabies virus (Flury-HEP) infected hamster cell culture (Nil-2) at 12 hours, 18 hours and 24 hours following infection.



OTHER PRODUCTS FROM INFECTED CELLS

In addition to infectious virus, a variety of other virus-induced products may be identified in harvests from cells infected with rabies virus.

Soluble antigen

The medium harvested from infected cells contains large quantities of non-infectious material with the immunological specificity of the G protein. It seems likely that the antigen is excess G protein which does not become incorporated into virus particles. This has the capacity to protect animals against a virulent virus challenge. This protective ability suggests the possible usefulness of soluble antigen in the preparation of a vaccine which would be free of rabies virus RNA. This antigen can be separated from the virion by the precipitation of the virion by zinc acetate, by ultrafiltration (30).

Defective interfering particles

Like many of the other rhabdoviruses, rabies virus has the ability to generate morphologically distinct RNA-containing defective interfering (DI) particles of varying sizes, many of which interfere with virus replication. They are considered to be deletion mutants and may be involved in the maintenance of persistent infections, both in tissue culture and animals. However, Wunner and Clark have been unable to find any correlation between their production and the expression of virulence (121). These particles are formed when multiplicity of infection (MOI) is high. They are defective in various viral functions, and must depend upon infectious virions to complement their

deficiency. Since their genomes replicate rapidly, these particles compete efficiently with normal genomes for encapsidation into the virion.

Interferon

In most cells infected with rabies virus, little or no specific damage was observed and the cells maintained in an actively growing state for long periods by regular division and feeding of the cultures. The maintenance of such a chronically infected state in BHK-21 cells has been attributed by workers at the Wistar institute, to the cyclic production of interferon, a protein produced by infected cells which interferes with virus growth. In their cell system, periodic cycles of low and high virus production appear to be related to the concentration of interferon in the culture (122). Cells in tissue culture can be effectively protected from rabies virus infection by treatment with exogenous interferon. This has been demonstrated in the cells of dog, hamster, mouse and human origin. Virus inactivated by ultraviolet light or by β -propiolactone did not induce interferon (31). Induction of interferon depends on the input of MOI.

RABIES VIRUS STRAINS AND CULTURES

In this study, we included fixed rabies strains such as Challenge virus standard (CVS), Paris strain, Pitman-Moore (PM), Flury High Egg Passage (HEP), Flury Low Egg Passage (LEP). Also two street strains isolated from human post-mortem brain samples were included.

CVS, Paris, PM strains were originated from Pasteur laboratory strains. The Paris strain was isolated from a rabid cow in France in 1882. This strain was adapted over 300 passages in rabbit brain; in cell culture, it was adapted in Vero cells. This strain was used to prepare β -propiolactone inactivated sheep brain vaccine. Pitman-Moore was originated by an adaptation of Pasteur virus in rabbit brain. Also it was cultivated in human diploid, primary dog kidney, Vero and Nil-2 cells. This strain was used in the preparation of dog kidney cell vaccine and the purified Vero cell vaccine for humans (123, 124). The CVS strain could be adapted in cell cultures such as mouse neuroblastoma cells (MNA) cells, BHK-21 cells and CER cells. This cell culture adapted CVS could be used in various in vitro neutralization tests (125). Since CVS is a derivative of the Pasteur strain, it is recommended as the challenge strain for the NIH test for testing vaccine potency. Earlier the suckling mouse brain vaccine was prepared using the inactivated CVS strain for the immunization of dogs (126).

Flury HEP and Flury LEP strains were originated from Flury strain which was isolated from a human in Georgia, USA, in 1939. These strains were used to prepare live attenuated vaccine. The Flury LEP strain was obtained by 40–50 passages in chick embryo fibroblast cells, whereas Flury-HEP was obtained

by over 180 passages. The Flury-LEP vaccine was used for the immunization of dogs. At 40th–50th passage level the virus was found to be pathogenic for mice, rats and hamsters when inoculated by intracerebral or intramuscular route. On the other hand the virus did not show any signs of infection in rabbits and dogs when given by intramuscular route. Further modification of the Flury-HEP has been achieved by continued egg passage (180th passage) and this has reduced its pathogenicity for experimental animals while its antigenicity has been retained. This vaccine was recommended for cattle and cats (127, 128). In 1957 Fox J et al. carried out a study on antirabies immunization of man using Flury-HEP vaccine. From their study it was found that though the strain was highly attenuated, it might safely induce immunity in man and the vaccine would be safe for immunization in man (129).

The two street samples used in the study were obtained from human post mortem brain sample from Maharashtra district. Both samples were obtained from individuals who were reported with case history of dog bite.

PURIFICATION OF RABIES VIRUS

Previously, a great amount of work has been done to study the structural, physical, chemical and antigenic composition of rabies virus. These studies required large amounts of homogeneous virus preparation, free from non-viral or cellular impurities. The main aim of the virus purification was for vaccine preparation. Concentration techniques have been applied to concentrate and partially purify the virus; however, such preparations showed the presence of cellular or other high molecular weight impurities, which have to be removed by appropriate techniques. Following are the techniques for the purification of rabies virus as well as its subunits shown by Schneider et al. (130).

Filtration and Chromatography

Filtration of preconcentrated rabies virus through sephadex G-75 has been used prior to the density gradient centrifugation in order to remove low molecular weight impurities (Zinc ions, EDTA) and a part of serum components of the tissue culture medium. For this purpose sephadex (particle size 40–120 μm) were equilibrated with Tris buffer, pH 7.8. The capacity of the column allowed the filtration of about 10 ml of virus.

Diethylaminoethyl (DEAE) cellulose chromatography was used as intermediate purification step in order to remove serum proteins or albumins. These substances were quantitatively eluted when the column of DEAE cellulose was developed with 0.15 M NaCl (130).

Density Gradient Centrifugation

In this technique, the particles sediment through a liquid column of a salt or a sucrose gradient by using high speed centrifugation. The particles are separated into zones according to their size, shape and density. The concentrated virus is either layered on top of the gradient or mixed with the solute and the gradient is allowed to form during centrifugation. After varying centrifugation times the particles are resolved in zones and are made visible as light scattering bands in a beam of light. Then the fractions are collected by puncturing the bottom of the tube with a hypodermic needle.

In rate zonal centrifugation, the concentrated virus is layered on sucrose gradient. A 10–60% gradient is centrifuged in swing bucket rotor for 90 minutes at 61,000 g. the virus particle band comes in the middle of the gradient. For isopycnic density gradient centrifugation Caesium chloride (CsCl) is mixed with pre-concentrated virus and centrifuged in the SW39 rotor at 1,00,000 g for 16–20 hours. The virus has been reported to band at a CsCl density of 1.20 or 1.22. These bands are then subjected to dialysis in an appropriate buffer (131).

Purification of rabies virus subunits

The purification of rabies virus subunits may be achieved by procedures similar to those described for complete virus particles. The RNP is separated from complete, disrupted virus by rate zonal centrifugation in sucrose gradient (10%–28% w/w) at 22,500 rpm for 2 hours 4°C. Also it is subjected to isopycnic density gradient centrifugation at 40,000 rpm to 25 hours.

The isolation of RNP of rabies virus from infected tissue culture cells was achieved by chromatography on sepharose 4B column by Schneider et al (130).

Purification of G protein is achieved by isopycnic sucrose density centrifugation. Sucrose gradients of 3 zones are used, bottom 3% sucrose, then 5–25% sucrose and on top 3% sucrose; which is centrifuged at 1,50,000 g for 36 hours at 4°C. The bottom fraction is collected.

Also the rabies virus proteins can be effectively separated and purified by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis under denaturing condition (132).

Hence on this background, the present study was designed to produce anti-RNP and anti-G MAbs and to study their reactivity against selected fixed and street rabies strains by indirect immunofluorescence assay.

AIMS & OBJECTIVE

RATIONALE FOR THE PRESENT STUDY

A few unexplained vaccine failures reported till date, may be due to the inadequacy of the antibodies to neutralize the infecting virus. Also the host system might be non-responsive to the vaccine, or a rabies related virus might be the cause of infection. In some cases, due to antigenic difference between street and vaccine strains of rabies, vaccine failure has been occurred. So it becomes necessary to find the antigenic difference between vaccine and street strains of rabies viruses. This is possible by using the monoclonal antibodies.

The diagnosis of rabies infection in humans and animals is carried out using antisera. These antisera do not permit to distinguish infections caused by vaccine or street strains of rabies viruses. As per WHO expert committee on rabies, indirect immunofluorescence assay (IIF) is more specific, sensitive and rapid diagnostic test. Due to the presence of common antigens, the use of polyclonal sera got limitations to differentiate virus variants. However, using MAbs produced against rabies viruses can differentiate between these variants. In addition, for epidemiological evaluations these MAbs are an important tool to investigate the prevalence of rabies viruses by using FA test directly on brain smears. Also serum samples can be tested for the presence of antirabies antibodies by using IIF test.

AIMS and OBJECTIVES

- *In vivo* and *in vitro* cultivation of following rabies virus strains.
 1. Fixed strains: Paris strain, Pitman-Moore (PM), Flury High Egg Passage (HEP), Flury Low Egg Passage (LEP).
 2. Street strains: Street I, Street II
- Maintenance and titration of Challenge Virus Standard (CVS) strain.
- Purification of Ribonucleoprotein (RNP) of rabies virus.
- Production of Anti-rabies monoclonal antibodies (MAbs).
- Purification of MAbs.
- Determination of total protein concentration of MAbs.
- Application of raised MAbs for differentiation of following rabies strain using Indirect Immunofluorescence assay.
 1. Fixed strains: Paris strain, Pitman-Moore (PM), Flury High Egg Passage (HEP), Flury Low Egg Passage (LEP).
 2. Street strains: Street I, Street II
 3. Standard challenge virus: CVS.

Due permission was obtained from the animal ethical committee of the Haffkine Institute for Training, Research and Testing, before the start of the project.

MATERIALS & METHOD

MATERIALS AND METHODS

1. Rabies virus strains used in the study

- **Standard challenge virus:** CVS was available from the preserved stocks in the Department of Virology, Haffkine Institute. This strain was cultivated in primary baby hamster kidney cell cultures. Also it was maintained by intracerebral inoculation in Swiss albino mice.
- **Fixed strains:** Paris strain, Pitman-Moore (PM), Flury High Egg Passage (HEP), Flury Low Egg Passage (LEP), were obtained from the preserved stocks from the Department of Virology, Haffkine Institute. These strains were maintained as mouse brain suspension in the vapour phase of liquid nitrogen.
- **Street strains:** Street I, Street II were isolated from post-mortem human brain samples and obtained from Bombay Veterinary College Parel, Mumbai.

2. Titration of Challenge Virus Standard (CVS) strain

Mice: Swiss Albino mice weighing 12–14 gm (Kasauli strain) were procured from Haffkine Biopharmaceutical Corporation Ltd. Mumbai and they were fed and watered *ad libitum* and maintained with proper ventilation.

The challenge virus was stored as a 20% suspension or as the supernatant following low speed centrifugation of suspension. The aliquots of the 20% suspension were prepared and stored at -80°C . Shortly, before the test was carried out, an ampoule was taken out from the stock and thawed rapidly under the tap. Then serial tenfold dilution was prepared using the diluent i.e., 2% Normal Horse Serum (2% NHS) inactivated at 56°C for 30 minutes.

The dilutions of the stock virus suspension (the tubes) were incubated for 1.0 –1.5 hours at 37°C . In order, to ensure that the conditions will be same as those of the serum virus neutralization step, the tubes were removed from the incubator and chilled in an ice-water bath. The groups of mice weighing 14–16 gms were inoculated intra-cerebrally with 0.03 ml of each virus dilution and observed for 14 days. The results were recorded and the virus titre was calculated using the Reed and Muench method (133).

Based on the above results, the LD_{50} of CVS strain was determined before the purification of ribonucleoprotein.

3. *In vitro* cultivation and purification of rabies virus

3.1 Growth and Maintenance of BHK-21 cell line

BHK-21 clone 13 cells susceptible to rabies virus were obtained from National Centre for Cell Sciences (NCCS, Pune). These were grown and maintained in Roux-flasks (1 L capacity) in the presence of 80–100 ml Modified Eagles base Medium (MEM; Hi-Media) and 10% Fetal calf serum (FCS; Hi-Media) (134).

The cells were routinely sub-cultured as described below. The roux flasks were drained off their growth medium. And the cell monolayer was washed couple of times with sterile phosphate buffered saline (PBS). One ml of 0.25% Trypsin Versene EDTA Glucose Phosphate (TPVG) was uniformly added on to the glass surface of the roux flasks. The Excess TPVG was drained out and the bottles were gently rotated for even spreading of the TPVG. Once the cells had started to detach from the glass surface, 5 ml fresh Eagles base medium with 10% FCS was added to the bottles. The cell clumps were separated by repeated pipetting using sterile glass pipette and then collected in sterile 20 ml culture vials (Laxbro) and washed three times by spinning down and resuspending in 10 ml Eagles medium containing 10% FCS. Fresh culture flasks were seeded with these cells at a count approximately 10^6 cells/ml. The flasks were incubated at 37°C until the confluent cell monolayer was formed.

3.2 *In vitro* cultivation of rabies virus

The rabies virus CVS was adapted and propagated on BHK-21 cells. The cells were infected with rabies virus in Roux bottles having capacity of 1 L and incubated for 1 hour at 37°C. The supernatant was discarded and the monolayer was rinsed with five ml of virus medium (MEM without FCS). The virus medium was then replaced with a fresh medium. The Roux bottles were sealed and incubated at 37°C for 60–72 hours in a humidified CO₂ incubator (3–5% Carbon dioxide). Cultures from the 20th passage were pooled to give a total volume of 800 ml. These pooled suspensions were then centrifuged at around 4000 g by using cooling centrifuge (Sorvall) for 10 minutes. The supernatant containing virus was then stored at -70°C till further use (135, 136).

3.3 Purification of rabies virus Ribonucleoprotein (RNP)

BHK-21 cells infected with the CVS virus were scraped. After washing once in PBS (Phosphate Buffered Saline pH 7.2), approximately 2 ml of cell pellet was suspended in 5 ml of ice-cold Distilled water (D/w) containing Aprotinin (Sigma). The cells were homogenized in a glass homogenizer and centrifuged for 20 minutes at 1000 g. The supernatant was collected. The pellet containing the cell debris was suspended as described above and centrifuged for 20 minutes at 1000 g. The supernatant was collected and the centrifugation step was repeated. The supernatant from both the steps was combined and centrifuged for 10

minutes at 12,000 g. The supernatant was collected. Six grams of Caesium chloride (CsCl; Sigma Chemicals) was added to 15 ml of resulting supernatant. Caesium chloride was dissolved, mixed well and centrifuged for 18 hours at 1,50,000 g at 4°C in a SW50 I rotor. After centrifugation, the RNP band was collected using a 23-gauge needle, at a buoyant density of 1.31g/cm³. Four grams of CsCl were dissolved in 9 ml of NTE (0.1 mol/l NaCl, 0.01 mol/l Tris-HCl, 0.001 mol/l EDTA, pH 7.5) buffer containing 2% OGP (octyl-β-D-glucopyranoside, Sigma Chemicals). Four volumes of this solution were mixed with 1 volume of RNP collected and were centrifuged for 18 hours at 1,50,000 g at 4°C. The band was collected as described earlier and dialysed against PBS (pH 7.2) using dialysis bags (Hi-Media) with a 10,000 Dalton cut-off (136). Six Swiss albino mice were inoculated intracerebrally to check the infectivity of the protein. The purification of the RNP was confirmed by SDS-PAGE and serologic analysis using fluorescent antibody test. The total protein was estimated by Folin-Lowry method (137).

3.4 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

(SDS- PAGE)

Principle: Sodium Dodecyl Sulphate-Polyacrylamide Gel electrophoresis involves the use of SDS. SDS, being a strong anionic detergent is used in combination with β-mercaptoethanol (reducing agent) to dissociate the proteins. The latter breaks disulphide linkages resulting in primary linear structure of the protein, whereas SDS imparts a net negative charge. Migration of the polypeptides is independent of shape and charge but

separation is indeed based on the molecular weight / size. Thus, by using markers of known molecular weights it is possible to estimate molecular weight of polypeptides or proteins.

Materials:

- Acryl amide
- SDS
- Tris-HCl
- Ammonium per sulfate
- TEMED
- Glycine
- Coomassie blue
- Methanol
- Glacial acetic acid
- Glycerol
- β -mercaptoethanol

Procedure:

SDS-PAGE was performed using vertical slab gel apparatus and run at 150 V for 2 hours. The purified ribonucleoprotein sample was dissolved in sample lysis buffer containing 0.5 mM Tris, 10% SDS, 0.005% bromophenol blue and 21% Glycerol pH 6.8. Sample as well as markers of known molecular weights were then applied to 10% Polyacrylamide gel. Stacking gel of 5% acrylamide was used. After electrophoresis, the protein bands were stained using Coomassie Brilliant Blue (CBB-R 250). All the reagents and buffers used for SDS- PAGE are described in Appendix.

3.4 Total protein estimation of purified RNP by Folin-Lowry method

Principle: The principle behind the Lowry method of determining protein concentrations lies in the reactivity of the peptide nitrogen[s] with the copper [II] ions under alkaline conditions and the subsequent reduction of the Folin-Ciocalteu phosphomolybdicphosphotungstic acid to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic acids. First, a cupric/peptide bond complex is formed and then this is enhanced by a phosphomolybdate complex with the aromatic amino acids. The Lowry method is sensitive to pH changes and therefore the pH of assay solution should be maintained at 10–10.5 (137).

Materials:

- Standard: Bovine Serum albumin (BSA) (500 µg/ml conc.).
- Analytical reagents:
 - a) 50 ml of 2% sodium carbonate mixed with 50 ml of 0.1 N NaOH solution.
 - b) 10 ml of 1.56% copper sulphate solution mixed with 10 ml of 2.37 sodium potassium tartarate solution. Prepare analytical reagent by mixing 2 ml of (b) with 100 ml of (a).
- Folin-Ciocalteu reagent solution (1N) Dilute commercial reagent (2N) with an equal volume of water on the day of use (2 ml of commercial reagent + 2 ml distilled water).

The purified RNP was treated as per the protocol. The Optical density (OD) at 660 nm was measured with a colorimeter, the concentration was estimated by

comparing the OD values to a Standard Graph plotted by using BSA as standard.

The method is as follows;

Different dilutions of Standard BSA were prepared. The BSA range was 50-500 µg/ml. the total volume was 1.0 ml to each tube 5.0 ml of Alkaline copper sulphate solution (Analytical reagent) was added, mixed well and kept at room temperature for 10 minutes. 0.5 ml of Folin Ciocalteu solution was added to each tube and incubated for 30 minutes. Optical density of the test tube was taken at 660 nm (137, 138)

4. Preparation of anti-rabies monoclonal antibodies.

Materials:

- Mice: BALB/c and Syngenic mice were procured from ACTREC Kharghar.
- Media and reagents:
 - Dulbecco's Modified Eagles Medium (DMEM, Gibco)
 - Fetal Calf Serum (FCS, Hi-media)
 - Complete Freund's adjuvant (Sigma Chemicals)
 - Rabipur vaccine (Aventis Pharma Ltd)
 - Polyethylene glycol 4000 (PEG, Sigma Chemicals)
 - HAT selective medium (Sigma Chemicals)
 - HT medium (Sigma Chemicals)
 - Horse serum (Hi-media)
 - Phosphate buffered saline (PBS) pH 7.2

4.1 Immunization of mice

Ten to twelve week old female BALB/c mice were immunized by an intraperitoneal inoculation of purified RNP (10 µg) with complete Freund's adjuvant (Sigma Chemicals). Other group of mice was immunized by Rabipuv vaccine (1 dose/mouse, potency: 2.5 IU, Aventis Pharma Ltd.). A booster inoculation was given intravenously 3–4 weeks later. Spleens were removed 3–5 days later (139).

4.2 Culturing of mouse Myeloma (SP2/0) cell line

Myeloma Cell line, SP2/0 was procured from NCCS, Pune. It was maintained in DMEM supplemented with 10 ml of 100-mmol/l sodium pyruvate and 10% fetal calf serum in a humid 5% carbon dioxide incubator. One week before fusion, the cells were transferred to DMEM containing 10% horse serum to improve the fusion efficiency. The cells were maintained in stationary suspension culture at concentration of 1×10^6 cells/ml and 5×10^6 cells/ml for fusion respectively. All sera were heated at 56°C for 30 minutes to inactivate the complement (139).

4.3 Production of hybrid cells (Fusion)

The mouse was killed by cervical dislocation and its body was immersed in 70% ethanol. The peritoneal cavity was opened and the exposed tissues were washed with 70% ethanol. The spleen was removed and it was transferred in

Petri dish containing 10 ml of PBS and a sterile metal grid. Then it was disrupted by pressing through the grid. The cell suspension was transferred into a 15 ml centrifuge tube. Cells were centrifuged at 200 g for 10 minutes and washed twice in PBS. The pellet was resuspended in 5 ml sterile NH_4Cl (haemolysing solution) to remove the red blood cells. The tube was kept at room temperature for 5 minutes. Seven ml FCS was added and centrifuged at 200 g for 10 minutes. This was again washed twice with DMEM without serum. The above suspension was finally suspended in 10 ml DMEM without serum.

In the mean time the myeloma cells grown in DMEM-10 HS (DMEM with 10% Horse Serum) were centrifuged at 200 g for 10 minutes and washed twice and resuspended with DMEM without serum. The myeloma cells and spleen cells were mixed together in ratios of 1:1, 1:4 and 1:10. Then these cells were centrifuged together at 200 g for 5 minutes in 45 ml conical centrifuge tubes. The supernatant was discarded and the tube was tapped gently to spread the pellet. One ml of 50% PEG 4000 was added over a period of 1 minute and mixed gently by tapping the tube. The tube was then incubated at 37°C for 10 minutes with 1 ml DMEM without serum overlaying the tube. Two ml of DMEM without serum was then mixed gently. Another 4 ml of DMEM-20 FCS (DMEM with 20% FCS) was mixed to the above solution with the help of a pipette. In the same tube of suspension, again 20 ml of DMEM-10 FCS (DMEM with 10% FCS) was added and centrifuged at 200 g for 5 minutes. The supernatant was discarded and the pellet was resuspended in 100 ml of DMEM-10 FCS and from this 1 ml of the cell suspension was distributed into each well of the microtitration plate. The plates were incubated for 24 hours at

37°C in a humid 5% carbon dioxide incubator. To each well 1 ml of DMEM-10 FCS-HAT (2X) concentrate was added. In a week, 1 ml of supernatant was replaced with 100 ml of fresh DMEM-10 FCS-HAT. After 7 days, 1 ml DMEM 10 FCS-HT medium was added to each well. Once the colony grows in the well, the culture supernatant was tested or screened for the presence of MAb: (139, 140).

4.4 Screening of Hybridoma supernatants

- **Indirect immunofluorescent antibody test (IIF):**

Principle

In this assay, the primary antibody (prepared MAbs) is unlabelled and is allowed to interact with the antigen (BHK-21 cells infected with CVS strain which is coated on a solid phase. This antigen-antibody complex is detected with a fluorochrome-labelled secondary antibody. Immune complexes containing fluorescent-labelled antibodies can be detected by coloured light emission when excited by light of an appropriate wavelength. The emitted light can be viewed with a fluorescence microscope, which is equipped with a UV light source. In this study Fluorescein Isothiocyanate (FITC) labelled anti mouse immunoglobulin was used as the secondary antibody.

Materials:

- Rabies virus infected BHK-21 cells
- Anti-mouse immunoglobulin FITC conjugate (Sigma)
- Teflon coated multispot slides
- Acetone
- Phosphate buffered saline (pH 7.4–7.6)

Procedure:

The virus infected BHK-21 cell monolayer was trypsinized and resuspended in MEM without serum to get approximately 10^4 cells/ml. Ten microlitres of the cell suspension were added to each of the 12 well Teflon coated multislide slides. After air drying, the smears were fixed with chilled acetone at -20°C for 30 minutes and washed thrice in PBS pH 7.4–7.6. After air drying, 10 μl of the hybridoma culture supernatant were added to a well and slide incubated at 37°C for 30 minutes in a moist chamber. At the end of incubation, 3 washings each for 10 minutes, were given with PBS. Anti-mouse immunoglobulin FITC conjugate (Sigma) was added to each well in a 1:100 dilution and incubated at 37°C for 10 minutes in a moist chamber. The slides were washed again thrice for 10 minutes each with PBS. Slides were then mounted with 50% buffered glycerol and observed under fluorescence microscope (141, 145).

- **Enzyme Immunoassay (EIA):**

Principle:

For the detection of rabies virus antiglycoprotein antibodies, the Platelia Rabies kit of Bio-Rad France (Cat. No. 72200) was used according to the manufacturer's instructions. The test is based on the use of a solid phase prepared with the glycoprotein extracted from the inactivated and purified virus membrane and an enzymatic conjugate. Briefly, in the first step, the test samples as well as controls are distributed in the cups of microtiter plate. If neutralizing antiglycoprotein antibodies are present; they bind with the viral antigen fixed on the solid phase. The second step comprises addition of the conjugate, which in turn, binds with the IgG retained by the solid phase. The

presence of the immobilized enzyme on the complexes is revealed by incubation in the presence of the substrate after the elimination of the unbound fraction of the conjugate. Lastly a spectrophotometric reading is taken at 492 nm after reaction has been stopped. Comparison with a control serum titrated in IU/ml, provides the titer of the unknown sample in EU (unit equivalent to the international units).

Materials:

- 96 well purified glycoprotein coated microtitration plate
- Washing solution concentrated 10X (Wash solution concentrate 10X was diluted 10 times in distilled water.)
- Diluent, concentrated 5X (One volume of Diluent 5X was added to 4 volumes of distilled water.)
- Conjugate, concentrated 10X: *Staphylococcus aureus* protein A labeled with peroxidase (One volume of concentrated conjugate was added to 9 volumes of washing solution.)
- Substrate buffer and Chromogen
- Stopping solution: 4N Sulphuric Acid

Procedure:

The plate was washed with Tris NaCl buffer (pH 7.4) with 1% Tween 20. Supernatant samples and controls were diluted 1/100 using concentrated skim milk as a diluent. Hundred microlitres of each dilution was dispensed per well and the plate was incubated for 60 minutes at 40°C in a humid incubator with 5% CO₂. The plate was emptied and washed 3 times with wash buffer.

Then 100 µl of conjugate was added in each well and the plate was incubated for 1 hour at 40°C in a humid incubator with 5% CO₂. The plate was emptied and washed 4 times with wash buffer. Hundred microlitres of Chromogen Ortho-Phenylene Diamine-2HCl was added to each well and the plate was incubated for 30 minutes at room temperature in the dark. Fifty microlitres of 4N Sulphuric acid was added as a stopping solution. The absorbance was read in a spectrophotometer at 492 nm.

4.5 Cloning of Hybridoma

After screening, the positive hybridoma colonies were separated and cloned by limiting dilution method using cloning medium (One part of myeloma culture supernatant and 2 parts of DMEM-20 FCS). After 10–15 days positive clones were selected using screening assays described earlier (139).

4.6 Bulk production of MAbs

- ❖ The monoclonal antibodies were produced by injection of hybridoma into the peritoneal cavity of syngenic mice. Approximately, 2×10^6 hybridoma cells in 0.5 ml of sterile saline buffer were injected into the peritoneal cavity of 12–20 week old syngenic mice. After 12–15 days the peritoneal cavities were tapped and the mice were sacrificed. The ascitic fluid were collected and centrifuged at 200 g for 10 minutes to obtain the supernatant (139).

- ❖ Hybridoma cells were inoculated into cell cultures at a concentration of 2.5×10^6 cells/ml. after 6 days, the supernatants were centrifuged at 200 g for 10 minutes, tested for desired antibody production and frozen at -20°C (139).

5. Purification of MAbs using Ammonium sulphate precipitation

Forty percent saturated solution of $(\text{NH}_4)_2\text{SO}_4$ was prepared for the precipitation of MAbs. Twenty millilitres of medium containing MAbs were mixed with 20 ml of saturated solution of $(\text{NH}_4)_2\text{SO}_4$ by stirring at 4°C – 8°C for 30 minutes. This was then centrifuged at 5000 g for 30 minutes. The pellet obtained was then dissolved in 40 ml D/w. The resulting solution containing MAbs was subjected to dialysis against PBS pH 7.2 using dialysis bags with a 10,000 Dalton cut-off (142).

The total protein concentration of each MAb was determined by the Folin Lowry method as described in 3.4.

6. Determination of the specificity of the monoclonal antibodies

Indirect immunofluorescent antibody test:

The specificity of anti-ribonucleoprotein MAbs (Anti-RNP) and anti glycoprotein MAbs (Anti-G) was carried out on rabies infected BHK-21 cells and on infected brain smears. The method is described in 4.4.

For this test, the following rabies virus strains were used.

- Fixed strains: Paris strain, Pitman-Moore (PM), Flury High Egg Passage (HEP), Flury Low Egg Passage (LEP) and Challenge Virus Standard (CVS) strain.
- Street strains: Street I, Street II.

All the samples were tested twice against MAbS produced to confirm the results

OBSERVATIONS & RESULT

RESULTS

1. Maintenance of rabies strains

In this study, total seven rabies strains were included. We have maintained following rabies virus strains by intracerebral inoculations into the Swiss albino mice. For long term preservation, the brain samples of infected mice were stored in liquid nitrogen in the form of homogeneous suspensions in 2% normal horse saline.

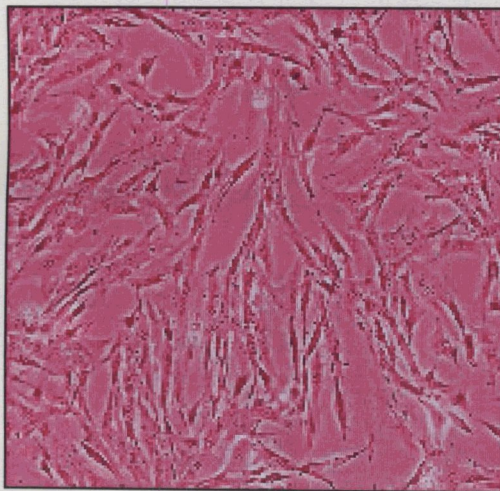
- Standard challenge virus: CVS.
- Fixed strains: Paris strain, Pitman-Moore (PM), Flury High Egg Passage (HEP), Flury Low Egg Passage (LEP).
- Street strains: Street I, Street II.

All the above strains were passaged once through the intracerebral route in Swiss albino mice. The brain tissues of the infected mice were used in the indirect immunofluorescence assay.

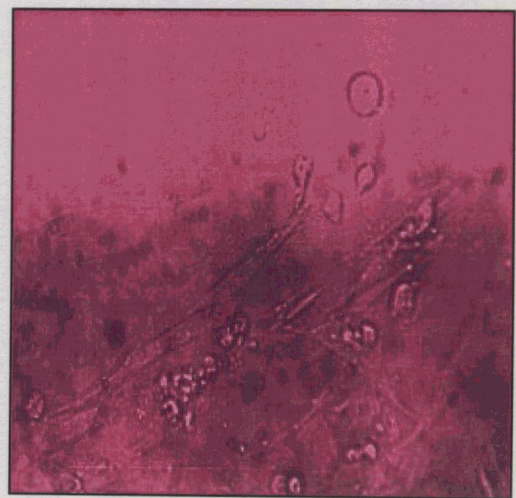
All the fixed strains were cultivated in BHK-21 cell line since the cell line is susceptible to these strains. But both the street strains were failed to grow in cell culture.

2. Growth and maintenance of CVS strain

For *in vitro* cultivation of CVS strain we have used monolayers of BHK-21 cell line. The cell line was maintained by using Modified Eagles base medium at a basic pH 7.4-7.6. In the infected BHK-21 cell monolayers, the cells merely began to age and detached more quickly from the supporting surface than uninfected control cells. Rabies virus was found to be released in the BHK-21 cell line supernatant, after 20 reinfections. The LD₅₀ of the virus was found to be 10^{4.3} LD₅₀/ 0.03 ml.



A



B

Figure 10.

- A. Healthy BHK-21 cell line (100X) maintained in MEM with 10% FCS.**
- B. Rabies virus infected BHK-21 cells showing CPE.**

3. Purification of rabies virus ribonucleoprotein (RNP)

The ribonucleoprotein was isolated from BHK-21 cells infected with CVS by isopycnic ultracentrifugation using CsCl as a gradient. The concentration of the protein after dialysis was found to be 4.6 mg/ml. The protein estimation was carried out by the method of Lowry et. al, by keeping bovine serum albumin as a reference.

The purity of RNP was confirmed by SDS-PAGE. Only a single band was observed when the purified RNP was subjected to SDS-PAGE. Thus the purified RNP was not found to be contaminated with other polypeptides.

Six swiss albino mice injected with purified RNP were survived, showing the safety of the antigen for immunization. The mice were injected intraperitoneally with the purified RNP. The anti-RNP serum was collected for the serological analysis using fluorescent antibody test. The results revealed the presence of antibodies against the RNP of the virus. This anti-RNP serum could not recognize the membrane antigen (G protein). Thus the isolated RNP was proved to be free of virus membrane material and cellular contaminants. This purified protein was used as one of the antigens in the study.

4. Production and Screening of hybridoma cells

In this study we carried out two different fusions for the production of anti-ribonucleoprotein (anti-RNP) MAbs and anti-glycoprotein (anti-G) MAbs. BALB/c mice were immunized with the selected antigens as per the schedule described by M. Lafon. During the course of immunization, no adverse reactions such as anaphylactic or neuroparalysis were observed in immunized BALB/c mice. After four weeks (after the booster dose), the blood was collected from the retro-orbital plexus of the immunized mice. The antibody titers for those specific antigens were determined by mouse neutralization test. Mice with higher antibody titer were selected as spleen cells donors. The titer of anti-RNP antibody was found to be 4.45 IU/ml whereas the anti-G antibody titer was found to be 5.23 IU/ml.

The myeloma cell line SP2/0 was maintained in Dulbecco's modified eagle's medium. The single, round cells in the suspension were maintained at a concentration of 5×10^5 viable cells/ml. The culture from the log phase was selected for the fusion. The fusion of splenocytes and SP2/0 was carried out as per the protocol.

After 15 days of fusion, the surviving colonies in the selective medium (DMEM-10-HAT) were screened. To select the clones producing anti-RNP MAbs, we used Indirect Immunofluorescent Antibody Test (IIF) and the selection of clones producing anti-G MAbs was done by Enzyme Immunoassay (EIA).

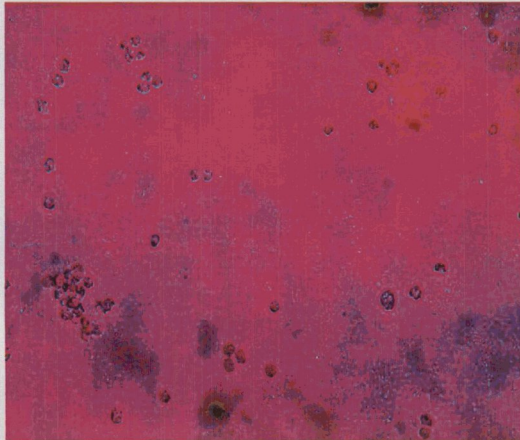


Figure 11. SP2/0 cell line growing in Dulbecco's modified Eagle's medium

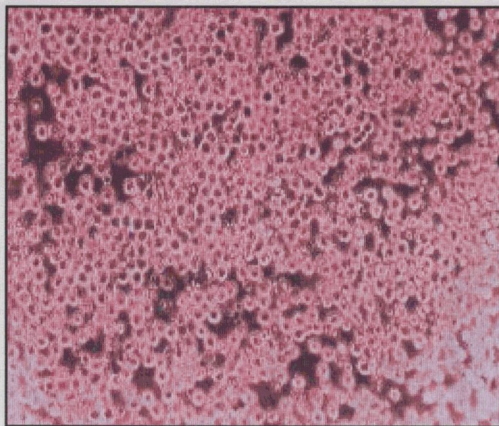


Figure 12. Growing hybridoma colony in DMEM-HAT medium

Screening of hybridoma supernatants

- **Anti-ribonucleoprotein MAbs (Anti-RNP MAbs)**

Respective supernatants from all the wells containing surviving colonies were subjected to screening by IIF assay. All the assay slides were observed under fluorescence microscope. The clones giving brightest intracytoplasmic fluorescence characteristic of the antibody to RNP antigen of rabies virus were selected for characterization and specificity study. Out of 42 surviving colonies, 20 colonies were positive. All the positive clones were cloned using cloning medium. Among these 20 colonies, only 5 colonies were selected, since these colonies were cloned twice by limiting dilution method and were found stable to produce respective MAbs.

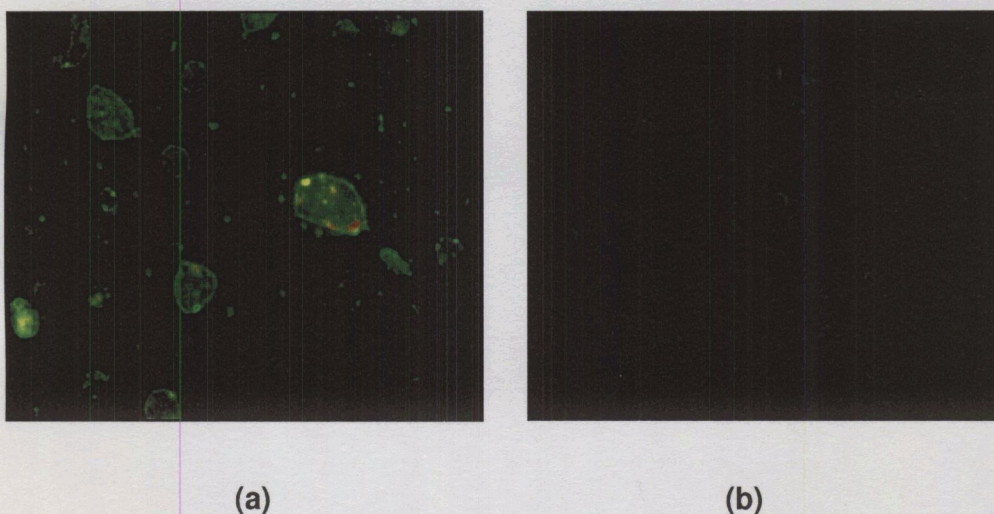


Figure 13.

(a) BHK-21 cells infected with rabies virus showing intracytoplasmic fluorescence in IIF assay in the presence of anti-RNP MAb.

(b) BHK-21 cells infected with rabies virus, in absence of anti-RNP MAb with no fluorescence.

- **Antiglycoprotein MAbs (Anti-G MAbs)**

Supernatants from the wells containing hybridoma colonies were screened by EIA. A specific absorbance at 492 nm equal to or greater than 0.1 was considered positive for anti-G antirabies antibody (143). Clones having >2.0 EU/ml concentrations of antibodies were selected for the study. These clones were subsequently cloned twice. Out of 52 surviving colonies, 30 colonies were found to be positive. From these colonies, 6 colonies were found to be stable after cloning and were selected for the study.

As mentioned in the pack-insert in the Platelia kit (Bio-Rad), we prepared the dilutions of positive control (standard antirabies antibodies). The EIA was carried out for these dilutions and OD was taken at 492 nm. These readings were used to plot a standard graph to get a standard equation for the respective kit. This equation was used to calculate the antibody titer of hybridoma supernatants under study.

Table 2: OD values for the Platelia EIA of standard antibody dilutions

Concentration of Positive control EU/ml	OD at 492 nm
0	0
0.075	0.078
0.15	0.081
0.3	0.107
0.6	0.148
1.25	0.266
2.5	0.492
5	0.912
10	1.496

Figure 12. Standard graph for anti-G Platelia EIA

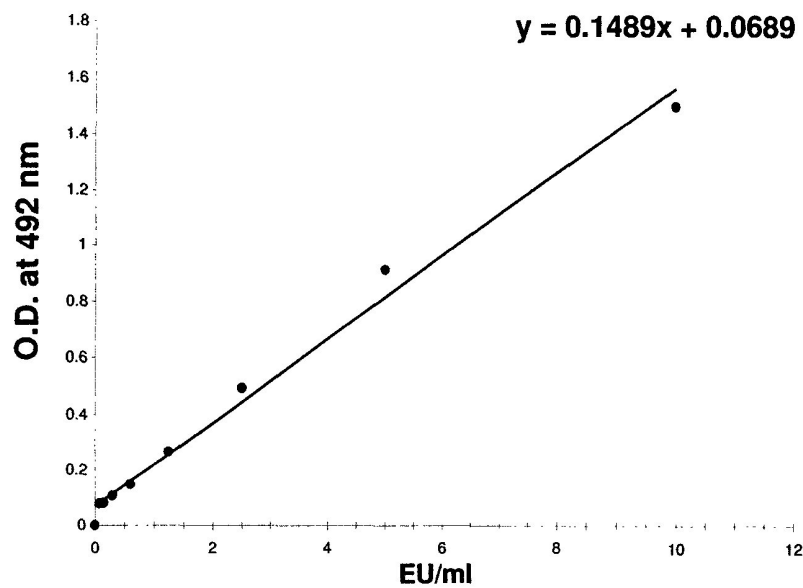


Table 3: Screening results of anti-G MAbs by EIA

Clone	Absorbance at 492nm	Antibody titer (EU/ml)
Positive control	1.210	7.7
A₁-1	0.458	2.6
A₅-1	0.431	2.4
A₆-1	0.479	2.8
C₃-1	0.422	2.3
C₃-3	0.523	3.0
D₄-3	0.448	2.5

(Anti-rabies antibody titer was calculated as per the pack-insert
in the Platelia kit: Bio-Rad.)

1 EU/ml $\approx$ 1 IU/ml

As described in the Table 3, selected clones producing anti-G MAbs gave the antibody titer more than 2 EU/ml. The clone C₃-3 gave highest titer i.e. 3.0 EU/ml. As per WHO, a titre of > 0.5 IU/ml is protective.

5. Cloning of hybridoma

After screening, the hybridoma secreting relevant antibodies were expanded in a second 24-well plate. These hybridoma were cloned twice by limiting dilution method to confirm that they are monoclonal.

6. Purification of Anti-RNP and Anti-G MAb

The prepared MAb were purified by Ammonium Sulphate precipitation method and were subjected to dialysis as described in materials and methods. all processes were carried out at 4°C. The protein estimation was carried out by the method of Lowry et. al, by keeping bovine serum albumin as a reference. The protein concentration of all the MAb was found to be more than 1.0 mg/ml.

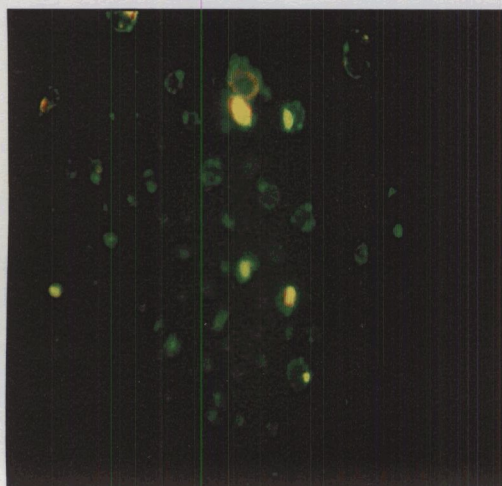
Table 4. Protein estimation by Folin-Lowry method

Total Protein (mg/ml)			
Anti-RNP MAb		Anti-G MAb	
A ₂ -2	1.68	A ₁ -1	1.08
B ₂ -2	1.58	A ₅ -1	1.76
B ₃ -2	2.57	A ₆ -1	1.25
B ₆ -2	1.58	C ₃ -1	1.36
C ₃ -2	1.10	C ₃ -3	1.11
-	-	D ₄ -3	1.48

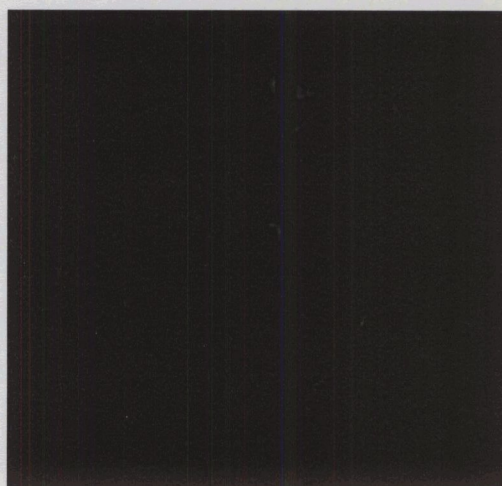
7. Determination of the specificity of the monoclonal antibodies

MAbs directed against RNP can be distinguished from those directed against G protein according to the localization of respective rabies antigens in infected cells. The slides were observed under the fluorescence microscope. The positive reaction was confirmed by the observation of brightly coloured, apple-green or greenish-yellow objects against a dark background. In case of negative reaction where there is no primary antibody against the coated antigen, the fluorescence was absent and slide appeared as dark.

Anti-RNP MAbs showed fluorescence in the cytoplasm of infected BHK-21 cells or neuron cells, whereas Anti-G MAbs showed membrane fluorescence. Based on the presence of fluorescence, reactions were classified as positive or negative. Some weak reactions were observed which were mostly considered as either negative or tested again with a different dilution of the respective MAbs.



(a)

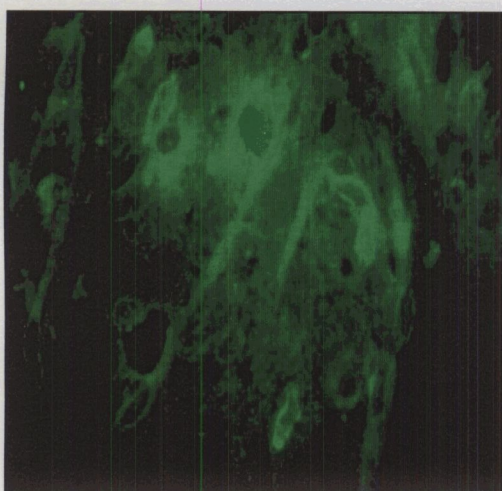


(b)

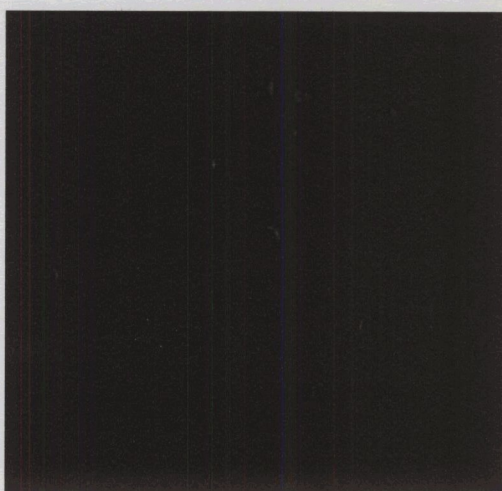
Figure 15. IIF assay for anti-RNP MAbs.

(a) Positive reaction showing intracytoplasmic fluorescence.

(b) Negative reaction showing no fluorescence.



(c)



(d)

Figure 16. IIF assay for anti-RNP MAbs

(c) Positive reaction showing membrane fluorescence.

(d) Negative reaction showing no fluorescence.

Table 5: Immunofluorescence reaction of 5 anti-RNP MAbs with

Anti-RNP MAbs	RABIES VIRUS STRAINS						
	CVS	PARIS	PM	HEP	LEP	Street I*	Street II*
A₂-2	+	+	+	-	+	+	+
B₂-2	+	+	+	+	+	+	+
B₃-2	+	+	+	+	+	+	-
B₆-2	+	+	+	-	+	+	-
C₃-2	+	+	+	+	+	+	+
Anti-N	+	+	+	+	+	+	+

rabies virus strains

+ = positive reaction - = no reaction * = street rabies samples from field isolates
 Anti-N = FITC labeled antinucleocapsid Anti-rabies antibodies

As illustrated in Table 5, all five clones producing anti-RNP MAbs gave positive fluorescence reaction against CVS, Paris, PM, Flury-LEP and Street I strains. The MAbs produced by clones A₂-2 and B₆-2 showed negative reaction against Flury-HEP strain and clones B₃-2 and B₆-2 gave negative reactions against Street II strain. Rest all clones gave fluorescence against Flury-HEP and Street II.

Table 6: Immunofluorescence reaction of 6 anti-G MABs with rabies virus strains

Anti-G MABs	RABIES VIRUS STRAINS						
	CVS	PARIS	PM	HEP	LEP	Street I*	Street II*
A₁-1	+	+	+	-	+	-	+
A₅-1	+	+	+	+	+	+	+
A₆-1	+	+	+	+	+	+	+
C₃-1	+	+	+	+	+	-	-
C₃-3	+	+	+	+	+	-	+
D₄-3	+	+	+	+	+	-	+
HRIG	+	+	+	+	+	+	+

+ = positive reaction

- = no reaction

* = street rabies samples from field isolates

HRIG = Human Rabies Immune Globulin

As shown in Table 6, all the six hybridoma antibodies gave membrane fluorescence against G protein of CVS, Paris, PM and Flury-LEP strains. However, when it came to reactivity with the G protein of Flury-HEP, Street I and Street II strains, only few MABs reacted against these viruses. The clone A₁-1 gave negative reaction against Flury-HEP and Street strains. The clone C₃-1 gave no fluorescence against both the street strains. Lastly, MABs produced by clones C₃-3 and D₄-3 failed to neutralize Street strain.

DISCUSSIO

DISCUSSION

Differentiating rabies virus strains was not possible until the pioneering work of Wiktor and Koprowski in 1978 and the production of first anti-rabies monoclonal antibodies (MAbs). Although the primary rationale for their development was to investigate the role of antigenic difference in vaccine failure, thereafter, it gradually became clear that MAbs could be used to distinguish rabies virus from other lyssaviruses. This technique could also be used to differentiate fixed from street rabies viruses and to discriminate among rabies viruses circulating in a variety of species throughout the world (17). The present study was aimed at producing anti-nucleocapsid (anti-RNP and anti-glycoprotein (anti-G) MAbs and testing their specific reactivities against Vaccine (Standard) and Street strains of rabies.

Monoclonal antibodies against the various antigenic determinants of the nucleocapsid and glycoprotein of rabies virus have been prepared in several WHO Collaborating Centers. These MAbs were used to identify wild strains of rabies virus originating from different geographical locations and reservoir species, as well as fixed strains used for rabies vaccine production (109, 110, 144). Many researchers have studied antigenic variations among the fixed strains, rabies related viruses and wild/street isolates from different hosts such as human, dogs, cats, cattles, bats, foxes, jackals etc. using MAbs.

In the present study we have used four fixed strains [Paris strain, Pitman Moore, Flury High Egg Passage (HEP), Flury Low Egg Passage (LEP)], one

challenge strain (CVS) and two Street strains (I and II). The specific reactivity of each strain under study was tested against MABs produced by us.

All the rabies strains used in the study were cultivated and preserved in the vapor phase of liquid nitrogen. The strains were inoculated in suckling mouse brain as well as in BHK-21 cell line, before their use in the Immunofluorescence assay. These brain tissue samples and infected BHK-21 cells were used in the assay to test the reactivity of the MABs produced. In the case of street strains it was found that, both the strains failed to grow in cell culture. This may be due to the inoculation of lower multiplicity of infection (MOI) of these strains in the cell culture (30).

We have cultivated the Challenge Virus Standard (CVS) strain *in vivo*, by using Swiss albino mice and *in vitro*, in BHK-21 cell line. Atanasiu et al. cultivated CVS strains in non-neural cells of a mouse ependymoma tumor cell line (116). Also this strain could be adapted in cell cultures such as mouse neuroblastoma cells (MNA) cells, Vero cells and Chick embryo related (CER) cells (125, 126). We preferred BHK-21 cell line, since it was found easy to maintain in a relatively simple medium and highly susceptible to the CVS strain.

Rabies virus was found to be released in the BHK-21 cell line supernatant after 20 re-infections. The LD₅₀ of the virus was found to be $10^{4.3}$ LD₅₀/0.03 ml. A similar study by Cokic-Danjanovic J. et al. showed the release of virus in supernatants of infected BHK-21 cells even after 100 passages without re-infection (120).

For the production of MAbs, we have used two purified antigens namely, ribonucleoprotein (RNP) and glycoprotein (G). In the present study the source of RNP was CVS strain whereas G protein was obtained from Rabipur vaccine. The RNP of CVS was isolated and purified by using isopycnic density gradient centrifugation technique, on a laboratory scale. After centrifugation the band of our interest was observed at a buoyant density of 1.31g/cm^3 . After centrifugation the RNP band was subjected to dialysis to remove excess of salts and impurities of low molecular weight. This purified RNP was injected intracerebrally into Swiss albino mice to check the activity of the virus. All mice withstood the purified RNP without infection. The result revealed the safety of the antigen for immunization. The SDS-PAGE result of purified RNP showed the appearance of single band which was comparable with standard molecular weight marker. This result showed the purity of the antigen. Immune sera prepared against RNP contained specific antibodies against virus RNP, which did not show reactivity against viral membrane proteins. Thus the RNP was proved to be free of viral membrane material and cellular contaminants. This method has advantage that RNP can be isolated in milligram amounts from infected tissue culture cells.

The isolation and purification of the rabies and several other rhabdoviruses from infected tissue culture cells was achieved by chromatography on Sepharose 4B by Schneider et al. (130). According to the work of Dietzschold the rabies virus RNP can be effectively separated and purified by SDS-PAGE under denaturing conditions (132).

In the present study, Rabipur vaccine (Purified chick embryo cell culture vaccine) was used as a source of G protein. This vaccine is commercially

available, marketed by Aventis Pharma Ltd. Rabipur vaccine is a pure vaccine with no detectable impurities as compared to other cell culture vaccines. The potency of this vaccine is 2.5 IU/ml. While Kapil A. et al. used Vero-Ral vaccine (Purified Vero cell rabies vaccine) for the immunization of mice (145),

The hybridoma technology was carried out to produce MAbs. The production of murine MAbs is well established technique, originally pioneered by Kohle and Milstein. We followed a protocol by Lafon M. and Zola H. by making a few experimental modifications (139, 140). Preparing MAbs requires we equipped tissue culture laboratory and an animal house. All these facilities were available in the Haffkine Institute. In this study, we have produced MAb by the fusion of mouse myeloma cells (SP2/0) and B cells from BALB/c mice immunized with purified RNP protein and G protein individually. Total 11 MAbs were studied of which 5 were anti-RNP MAbs and 6 were anti-G MAbs.

There are many protocols for the immunization of animals (mice) (139). The best immunization procedures clearly depend on the antigen in question. In the present study, purified antigens were used for immunization in order to obtain homologous antibodies against them, so that screening and selection of positive clones becomes easier. Further, this avoids an immune response against contaminants as well. Both of these antigens were injected individually along with the complete Freund's adjuvant in BALB/c mice. The boosters were given according to the schedule (139). The antibody titers were monitored and mice producing high titers of antibodies were selected for the experiment. It was found that the immunized mice produced antibodies with :

high titer against the respective antigens without any adverse reactions such as anaphylactic reactions or neuroparalysis.

Wiktor and Koprowski (1980) used concentrated, purified β -propiolactone inactivated vaccine prepared from the ERA, CVS, Kelev or Mokola strains for mice immunization (96). Whereas Dietzschold B. et al. produced human MAbs from peripheral B lymphocytes from vaccinees immunized with HDCV by somatic cell hybridization (146). From the other studies it can be seen that the researchers have used either purified, inactivated whole virus or commercially available tissue culture vaccines for immunization in hybridoma technology.

In order to be suitable for hybridoma work, a myeloma must fulfill certain criteria. It must grow well, hybrids derived from it must grow well, and they must secrete high concentrations of immunoglobulin. Unlike lymphocytes and hybridomas, these cells are unable to grow in selective HAT medium. In the present study sublines of myeloma that do not produce heavy or light chains SP2/0 was chosen in order to facilitate screening and purification procedures. Myeloma cells are susceptible to mycoplasma contamination. Myeloma cultures were checked regularly for mycoplasma contamination and contaminated cultures were discarded. Previously, Wiktor and Koprowski have used P3X63-Ag8 myeloma cell line. However this cell line produces its own immunoglobulin and the fusion product can secrete mixed molecules consisting of heavy chains from one parent and light chains from other. Therefore SP2/0 cell line was chosen and was found to produce fewer hybrids than P3X63-Ag8 (147).

Fusion of two different cell membranes can occur with a very low frequency when cells are brought together, and the frequency can be increased by the addition of fusogens. The established fusion protocols use polyethylene glycol (PEG) to induce membrane fusion. Successful fusions have been reported with batches ranging in nominal molecular weight from 500 to 6000 (131). We have carried out all the fusions using PEG 4000. Electroporation and electroacoustic techniques are alternatives that are useful when low numbers of specific B cells are available (140). The fusion technique should complete rapidly, otherwise it can cause damage to the cells. The success of the fusion depends on cell ratio, medium used, conditions for achieving contact, fusing agent, conditions for fusion (time, temperature, physical handling) and procedure after fusion up to the plating out stage. By considering all these factors we have carried out two different fusions for anti-RNP and anti-G MAb production. We have used the original protocol with few variations viz. after fusion; the fused cells were distributed in 24 wells microtitration plates instead of 96 well plates, many protocols showed the application of macrophage feeder layer before the inoculation of fused cells into the plates (139). Improved yields of hybridoma are obtained by addition of feeder cells. But in this study, we have found that the hybridoma colonies grew well even in absence of the macrophage feeder layer.

One of the keys to successful development of MAbs is the screening assay. The more specific and simple the screening test, the better the chance of obtaining MAbs of interest. After fusion, we observed large number of growing colonies in the experimental plates after 8–10 days. The medium in the culture began to turn yellow. On 16th day, the supernatants from the surviving

hybridoma colonies were screened. To select the clones producing anti-RNP MAbs, we have used Indirect Immunofluorescent Antibody Test (IIF) and the selection of clones producing anti-G MAbs was done by Enzyme Immunoassay (EIA). In IIF test, the supernatants producing anti-RNP MAbs were selected on the basis of positive fluorescence reaction as shown in Figure 13. Selection of anti-G MAbs was carried out by using Platelia EIA kit. According to the kit-insert, supernatants giving anti-G titer more than 2 EU/ml were considered as positive. As shown in Table 3, our selected clones gave titer more than 2 EU/ml. Since the EIA performed was quantitative, all the selected clones gave a protective titer according to the kit-insert. On this background, a protective study using these anti-G MAbs would be carried out in mice. These screening techniques were found to be very simple and easy to perform and also large numbers of supernatants could be screened in a short period of time. By these techniques, positive clones producing MAbs of interest were selected for expansion and cloning.

For screening, Wiktor and Koprowski used Radioimmunoassay (RIA) (17) whereas Indirect Immunofluorescence Assay was used by Kapil et al. (145). The work of Lafon and Edelman shows the use of Enzyme Immuno Assay for analysis of MAb secretion (148).

Cloning is an extremely important step, since the hybrids have a tendency to lose chromosomes randomly till they stabilize. Cloning was carried out to ensure a given culture contains only one cell type and that it is producing only one immunoglobulin specificity. Hybrids may become non-secretors during sub cloning due to loss of chromosomes. It was necessary to find all selected clones were stable after two consecutive limiting dilutions. In this study

clones which gave positive results in screening assay were selected. During all these steps care was taken to avoid mycoplasma infection in the cultures. Hybridomas suspected of contamination were rejected from the study.

After fusion, the total number of surviving colonies producing anti-RNP MAbs was 42. Out of 42 surviving colonies, 20 colonies were positive. Among these 20 colonies, only 5 colonies were selected, since these colonies were cloned twice by limiting dilution method and were found stable to produce respective MAbs.

Out of 52 surviving colonies producing anti-G MAbs, 30 colonies were found to be positive. From these colonies, 6 colonies were found to be stable after cloning and were selected for the further study. The above results showed that about 50% surviving colonies were positive; this may be due to the use of purified antigens for immunization in the beginning. Also this shows the specificity and sensitivity of the MAbs to the respective antigens used for immunization.

Large amounts of monoclonal antibodies have been produced by methods involving production of ascites fluid obtained by injecting hybridoma into peritoneal cavity of syngenic mice, and production of large volumes of hybridoma supernatants from cell cultures. However, due to the production of non-relevant antibodies present in the peritoneal fluid, which interfere in purification procedure, the cell culture technique was preferred, where the production of MAbs was found to be easier. The cells were removed from the harvested culture by centrifugation and the supernatants containing MAbs were subjected to purification and lastly stored at -20°C . Thus in this way we

obtained large volumes of MABs on a laboratory scale without using large scale culture apparatus.

Cryopreservation of hybridoma is an essential safeguard against loss of valuable lines. In the present study after screening most of the positive clones were preserved in liquid nitrogen. After cloning, the new clones were preserved in liquid nitrogen.

For many purposes cultures with MABs may be used without purification however it may be necessary to purify the MABs, for example for labeling with radioactive or fluorescent tags. Depending on the purity required, the MABs may be precipitated with ammonium sulphate or a higher degree of purity may be achieved by ion-exchange chromatography or affinity separation. In the present study, ammonium sulphate precipitation was carried out at 4°C to avoid possible chances of bacterial contamination during the purification of MABs. The advantage of this method is that it is simple, removes unwanted proteins maximally and gives high percentage of recovery in a single step. Schumacher et al. had purified MABs by using HPLC ion exchange chromatography (112). In our study the total protein concentration of the purified MABs was found to be more than 1.0 mg/ml.

After the successful production and purification of anti-RNP and anti-G MABs the most important parameter of the study was carried out. In the present study we present a panel of MABs which can identify and differentiate selected rabies strains. The specificity of MABs to various rabies viruses was checked using Indirect Immunofluorescence Assay. Each virus sample was

processed with the MAbs panel at least twice in different batches to determine the reproducibility of the test. Samples showing weak positive reaction were processed in duplicate with different dilutions of respective MAbs. Acetone fixed smears of BHK-21 cells containing virus as well as non-fixed cells were used for IIF assay. In this study we have found that IIF assay can be done directly on brain samples preserved at -70°C . Anti-RNP MAbs showed fluorescence in the cytoplasm whereas anti-G MAbs showed membranous fluorescence (as described in Figure 15 and 16). Based on the presence of fluorescence, reactions were classified as positive or negative. The technique was found to be very simple to perform and cost effective.

Anti-RNP MAbs: As per table 5, MAbs secreted by hybridomas B₂-2 and C₃-2 show similar specificities for all selected virus strains. But the epitopes recognized by the clones A₂-2 and B₆-2 are not present on Flury-HEP strain. In addition, the clone B₃-2 and B₆-2 did not neutralize the epitopes on street-I strain. Here the surprising fact is that the MAbs A₂-2 and B₆-2 did not react with nucleocapsid of the Flury-HEP virus despite the fact that the HEP virus is a derivative of Flury-LEP. Thus, these results reveal that in the IIF assay anti-RNP A₂-2 and B₆-2 allows us to distinguish between Flury-HEP and the other rabies strains maintained in the laboratory. Also street II strain can be differentiated by using B₃-2 and B₆-2 MAbs.

Anti-G MAbs: As per table 6, Hybridoma A₅-1 and A₆-1 showed specificity to all selected strains. However, A₁-1 does not neutralize HEP virus as well as street-I strain. Similarly MAbs produced from clones C₃-1, C₃-3 and D₄-3 do not recognize street-I strain. Also the epitope on Street-II strain was not

recognized by clone C₃-1. Thus anti-G MAbs A₁-1, C₃-1, C₃-3 and D₄-3 can be used as a diagnostic tool to differentiate rabies strains. Since four clones in the study failed to neutralize the epitopes on street strains, which might be one of the reasons of vaccine failure.

According to Wiktor and Koprowski, vaccine failure correlated with the degree of antigenic disparity between vaccine and challenge viruses as revealed by analysis with MAbs (149).

MAbs produced by hybridomas expressed different specificities in interaction with various strains of rabies virus. A₂-2, B₃-2, B₆-2, A₁-1, C₃-1, C₃-3 and D₄-3 panel of hybridoma antibodies not only provides a tool for the diagnosis of rabies in the tissue of infected subjects but also permits spot viral antigen classification. Also this panel can be useful in investigating origins of viruses involved in post vaccine failures.

According to the work of Wright et al., antigenic differences in nucleocapsid protein divide the vaccine strains into subgroups. The vaccine strains ERA, SAD and LEP, HEP, can be distinguished from each other and from the strains CVS, Pasture virus and PM. Also anti-glycoprotein MAbs distinguish Pasteur vaccine strain from other strains (150). Wiktor and Flamand showed the antigenic difference of RNP between selected rabies strains and HEF strain. Also their work shows in FA test, MAb 377-7 stains nucleocapsids of a fixed and street strains studied except CVS (151).

From the study it can be seen that fixed rabies virus falls into antigenic groups in relation to its cross reactivity with antibodies produced by a given

hybridoma. All vaccine strains were recognized by the MAbs prepared by us in the panel except Flury-HEP strain. Results show the marked antigenic differences among the street strains and vaccine strains, which might be one of the causes of vaccine failure. This panel can be used to study the specificity as well as the discrimination of rabies virus isolated from different hosts in India. Since the anti-G MAbs produced by the method described earlier yielded antibodies more than 2 EU/ml in EIA, the protective study in mice can be done by using these antirabies MAbs against rabies viruses in further studies.

According to Schumacher et al., the cocktail of MAbs, consisting of anti-G MAbs and anti-RNP MAbs is highly effective in PEP (112). Such a cocktail has several advantages over hyperimmune sera, these MAbs can be used for local wound treatment. For the foreseeable future, however, there can be no doubt that MAbs will be an essential tool in identifying those factors that affect the maintenance and spread of rabies in nature.

Thus the main aim of the study, which was to produce anti-RNP and anti-G MAbs was successful. The findings from the study could contribute towards a cost effective technique for the diagnosis and differentiation of various rabies virus strains.

SUMMARY & CONCLUSIO

SUMMARY AND CONCLUSIONS

Following conclusions are made from the present study:

1. The present study was undertaken to produce anti-RNP and anti-G anti-rabies MAbs. Also the specific reactivity of these MAbs was checked against fixed strains viz. CVS, PM, Paris strain, Flury-LEP, Flury-HEP and street rabies strains I and II, by using indirect immunofluorescence assay.
2. The hybridoma technique was applied to produce MAbs by the fusion of mouse myeloma cells (SP2/0) and B cells from BALB/c mice immunized with either purified RNP or G protein. Total 11 MAbs were studied of which 5 were anti-RNP MAbs and 6 were anti-G MAbs.
3. The specificities of the MAbs were determined by using screening assays. All the MAbs were found to be specific for respective antigens (purified RNP and G) used for immunization.
4. From the indirect immunofluorescence assay results, it can be concluded that Flury-HEP may have certain different epitopes than other fixed strains. Few MAbs in the panel failed to neutralize these epitopes. Thus the panel of MAbs helped to differentiate Flury-HEP strain from other fixed strains. Also the two street samples showed antigenic variations for certain epitopes. Street samples were differentiated from fixed strains.

5. Indirect Immunofluorescence assay was found to be a good technique for diagnosis of Rabies Infection. In addition, the fact that this assay may be used for *in situ* diagnosis (i.e. directly on Brain Samples) provides additional flexibility.
6. In order to develop a logical basis for the selection of fixed strain for vaccine production, it is necessary to know the antigenic variations of rabies viruses circulating in India. This can be done by employing anti rabies monoclonal antibodies. In future these MAbs may be able to fulfil the global need for rabies prophylaxis.
7. The EIA results could be useful to understand more on the use of MAbs in PEP since all the anti-G MAbs produce antibody titer more than 2 EU/ml. Further, the protection study can be carried out in mice using these MAbs against various rabies strains. The cocktail of MAbs could be used for passive immunization.
8. Although the initial hybridoma technology is a complex, time consuming technique, proper skills and expertise will give promising results to produce required MAbs on large scale.

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APPENDI

APPENDIX I

1. Modified Eagles base Medium (MEM)

Powdered medium obtained from Hi-Media was dissolved in 900 ml Distilled water (D/w). pH was adjusted to 7.4–7.6 using 1N HCl and 1N NaOH. Following constituents were added:

L- Glutamine	: 2 mM
NaHCO ₃	: 1.5 g/L
Non-essential amino acids	: 0.1 mM
Sodium pyruvate	: 1.0 mM

Additional D/w was added to bring the volume to 1L. The medium was then filter sterilized using membrane with a porosity of 0.22 μ . The filtered medium was aseptically dispensed into sterile bottles and stored at 4°C.

2. Dulbecco's Modified Eagles Medium (DMEM, Gibco)

Content of one packet was dissolved in 900 ml of D/w. Following constituents were added.

NaHCO ₃	: 1.5 g/L
L-Glutamine	: 4 mM
Antibiotics	
a) Penicillin	: 100 Units/ ml
b) Streptomycin	: 100 μ g/ ml
c) Gentamycin	: 20 μ g/ ml

Total volume was made to 1000 ml with D/w and DMEM was sterilized by filtration. This is termed as Serum free DMEM, used for washing of cells used for fusion.

3. HAT selective medium

The vial of HAT supplement (50X Hybrimax, Sigma) was reconstituted with 10 ml of serum free DMEM with concentration of Hypoxanthine 5×10^{-3} , Aminopterin 2×10^{-3} and Thymidine 8×10^{-3} . Working medium of 1X was prepared in complete DMEM.

4. HT medium

Except Aminopterin all other constituents are same as that of HAT medium.

5. 50% Polyethylene Glycol 4000 (PEG)

0.5 gms PEG was autoclaved and to this 0.5 ml serum free DMEM was added and mixed properly. PEG was prepared fresh on the day of fusion.

6. Phosphate buffered saline (PBS) pH 7.2

Na₂HPO₄.2H₂O: 2.98 g

NaH₂PO₄ : 0.49 g

NaCl : 8.50 g

Above chemicals were dissolved in D/w and volume was made to 1000ml

APPENDIX II

1. NTE Buffer (pH 7.5)

NaCl : 0.1 mol/L

Tris-HCl : 0.01 mol/L

EDTA : 0.001 mol/L

Above constituents were dissolved in 1 L D/w and sterilized by Millipore filtration through 0.22 μ membranes. This buffer was stored at 4 °C.

2. 2% NHS

Two milliliters of inactivated Normal Horse Serum was diluted to 100ml using D/w.

3. Phosphate Buffered saline (pH 7.4–7.6)

NaCl : 8.00 g

KCl : 0.20 g

Na₂HPO₄ : 1.44 g

KH₂PO₄ : 0.24 g

Above chemicals were dissolved in 800 ml of D/w. the pH was adjusted to 7.4 with HCl. Then D/w was added to make the volume to 1 liter. This solution was sterilized by autoclaving.

Reagents for SDS-PAGE

1. Acrylamide Stock Solution (30%):

One gram of Bisacrylamide was added to 30 ml Distilled water and heated upto 50 °C to 70 °C to dissolve. After this, 29 g of acrylamide was

added and the final volume was made upto 100 ml. the solution was stored in brown bottle at 4 °C.

2. Stacking gel Buffer pH 6.8, 0.5M:

Six grams of Tris was dissolved in 40 ml D/w. The pH was then adjusted to 6.8 with 1 N HCl and the volume was made upto 100 ml.

3. Resolving gel buffer pH 8.8, 3M:

36.3 gms of Tris was added to 48 ml of D/w. The pH of the solution was adjusted to 8.8 and then the volume was made upto 100 ml using D/w.

4. Tank Buffer:

One gram SDS was added to the mixture containing 3 gms of Tris and 14.4 gms of glycine and the final volume was made upto 1000ml.

5. Gel Loading Buffer Preparation:

0.5 M Tris (pH 6.8)	1 ml
Glycerol	2 ml
β-Mercaptoethanol	1 ml
10 % SDS	4 ml
Bromophenol Blue (0.25 %)	2.5 mg
Distilled Water	1.5 ml

6. Stacking Gel (5% acrylamide) Preparation:

Ammonium per sulphate (APS)	40 µl from 10% stock
Distilled Water	2.7 ml
Stacking Gel Buffer	0.5 ml from stock
Acrylamide (5%)	0.67ml from stock

10 % SDS	40 µl
TEMED	4µl

7. Resolving gel (10 % acrylamide) Preparation:

Ammonium per sulphate (APS)	100 µl from 10% stock
Distilled Water	4.0 ml
Resolving gel Buffer	2.5 ml from stock
Acrylamide (10%)	3.3 ml from stock.
10 % SDS	100 µl
TEMED	4µl

8. Staining solution (Coomassie Brilliant Blue) Preparation:

0.25% Coomassie Brilliant Blue (CBB-R 250) was dissolved in Methanol:Glacial Acetic Acid:D/w (1:1:8 ratio) and filtered and stored in an amber colored bottle at room temperature.

9. Destain I

10% Methanol	30 ml
10% Glacial acetic acid	30 ml
D/w	120 ml

10. Destain II

50% Methanol	150 ml
10% Glacial acetic acid	30 ml
2% Glycerol	6 ml
D/w	234 ml

LIST OF ABBREVIATIONS

List of Abbreviations

ABLV	Australian Bat lyssavirus
APCRI	Association for Prevention and Control of Rabies in India
BHK-21	Baby Hamster Kidney 21 Cells
BSA	Bovine Serum Albumin
CDC	Centers for Disease Control and Prevention (USA)
CER	Chick Embryo Related
CNS	Central Nervous System
CVS	Challenge Virus Standard
DFA	Direct fluorescent antibody
DI	Defective interfering (particles)
DMEM	Dulbecco's modified Eagle's medium
EBL	European bat lyssavirus
EIA	Enzyme immuno assay
ELISA	Enzyme Linked Immunosorbent assay
ERIG	Equine rabies immunoglobulin
FAT	Fluorescent antibody test
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
HAT	Hypoxanthine-Aminopterin-Thymidine
HT	Hypoxanthine-Thymidine
HDCV	Human Diploid Cell Culture Vaccine
HEP	Flury high egg passage strain of rabies virus
HIV	Human Immunodeficiency Virus
HRIG	Human rabies immunoglobulin
IIF	Indirect immunofluorescent antibody test
IU	International Unit
LD ₅₀	Median lethal dose
LEP	Flury low egg passage strain of rabies virus
MAb	Monoclonal antibody

MAB-anti G	Anti-glycoprotein monoclonal antibody
MAB-antiRNP	Anti-ribonucleoprotein monoclonal antibody
MEM	Minimum essential medium
MNA	Mouse Neuroblastoma cells
MNT	Mouse neutralization test
NASBA	Nucleic acid sequence-based amplification
OD	Optical density
PAGE	Poly-Acrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCECV	Purified Chick Embryo Cell Culture Vaccine
PCR	Polymerase Chain Reaction
PEG	Poly ethylene Glycol
PEP	Post-exposure prophylaxis
PM	Pitman-Moore strain of rabies virus
PVRV	Purified Vero Cell Rabies Vaccine
RIG	Rabies Immunoglobulin
RFFIT	Rapid fluorescent focus inhibition test
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TEMED	<i>N,N,N',N'</i> - Tetramethelene diamine
TPVG	Trypsin Versene – EDTA Glucose Phosphate
VNA	Virus Neutralizing Antibodies
WHO	World Health Organization

SYNOPSIS

A SYNOPSIS OF THE THESIS
TO BE SUBMITTED TO THE UNIVERSITY OF MUMBAI
FOR
THE DEGREE OF MASTER OF SCIENCE
IN MICROBIOLOGY

Title of the thesis : Preparation Of Rabies Monoclonal Antibodies As A Diagnostic Tool For Rabies Strain Differentiation.

Name of the candidate : Ms. Swapnaja P. Ghadigaonkar

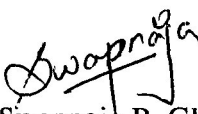
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PREPARATION OF RABIES MONOCLONAL ANTIBODIES AS A DIAGNOSTIC TOOL FOR RABIES STRAIN DIFFERENTIATION

Introduction

Rabies, a dreaded human disease, rampant in the developing world, was known as early as 2300 B.C. Greeks called rabies *Lyssa* or *Lytta* which means- madness. The Latin word 'rabies' is derived from Sanskrit word '*rabhas*' which means 'to do violence'. Rabies is transmitted from animal to animal or from animal to human by exposure to infected saliva. The virus attaches to peripheral nerve endings in the infected host and travels to the brain. All mammalian species are susceptible to rabies infection. Human infections with rabies are mostly secondary to animal bites. Although, the infection may also occur by the inhalation of virus, through the transplantation of infected corneas or from infected lactating mother to her child. ⁽¹⁾ As the virus travels quite slowly centripetally in the body, post exposure prophylaxis can prevent the death. But once the clinical symptoms of rabies have established, it is a 100% fatal disease. Intriguingly, once the rabies virus has entered the Central Nervous System (CNS), its progression is uninterrupted either by destruction of the infected neuron or by the immune response.

Rabies is becoming a re-emerging disease in several parts of the world including China where epidemics are currently exploding. Only a handful of countries protected by the natural barrier of sea around them or their island location are free of rabies. Britain is one of them, free of rabies for the last 50 years because of its strict quarantine regulations and the geological location. ⁽²⁾ According to WHO, more than three billion people are at risk for rabies in over 85 countries and territories worldwide, and about 50,000–60,000 human deaths from rabies are estimated to occur annually, with 30-60% of these cases being seen in young children. Even though effective vaccines for post-exposure prophylaxis (PEP) are available, over 10 million individuals actually receive rabies PEP each year. ⁽³⁾ Rabies remains an important yet neglected disease in Africa and Asia. The highest number of human rabies cases is reported in Africa and Asia, particularly the Indian sub-continent. Asia carries a larger part of the public health burden of rabies with an estimated 31,000

deaths, although the estimate for Africa (24,000 deaths) is much greater than initially believed. In 2003, National Survey by the Association for the Prevention and Control of Rabies in India estimated 18,500 human deaths from rabies in the country each year, out of which 89 per cent had not taken the post-exposure prophylaxis. In India 1.07 million patients receive post exposure treatment annually. ⁽⁴⁾ In most countries of Africa, Asia, Latin America, and the Middle East, dogs are found to be the main hosts and are responsible for most of the human rabies deaths that occur worldwide. In contrast, in North America, most documented human rabies deaths occurred as a result of infection from the silver-haired bat rabies virus variant. ⁽⁵⁾

Rabies virus belongs to the family *Rhabdoviridae*; genus *Lyssavirus* consisting of genetically related enveloped viruses with a single, non segmented, negative-stranded RNA. The *Lyssavirus* serogroup consists of six serotypes on the basis of serological and antigenic relationships: serotype one consists of 'classical' rabies viruses, notably the 'street' (wild) and vaccine strains. Serotypes 2, 3, 4, 5, 6 and 7 consist of several rabies-related viruses including *Lagos bat*, *Mokola*, *Duvenhage*, *European bat*, *Obodhiang Kotonkan* and Australian bat virus. These viruses have been isolated in different parts of the world and from a wide variety of hosts like bats and shrews. These viruses are not naturally found in India. ⁽⁶⁾ They share high levels of amino acid and nucleotide sequence homology with rabies virus and cause a clinical disease indistinguishable from rabies encephalitis. Specific identification is performed by using monoclonal antibodies (MAbs) and genetic typing. Also animals protected against rabies virus are not necessarily immune to infection with the rabies related viruses. ⁽⁷⁾

Rabies virus consists of following structural antigens which might behave as a unique antigen:

- RNA dependent RNA polymerase L protein (180 KD).
- Glycoprotein G, exists in two forms G I (65 KD) and G II (70 KD).
- Nucleoprotein N (57 KD).
- Phosphoprotein M1, exists in two forms M1 I (38 KD) and M1 II (41KD).
- Matrix or membrane protein M2 having two conformations M2 I (24KD and M2 II (25 KD).

These antigens carry one or more antigenic sites and are serotype specific and cross-reactions are seen with some rabies related viruses. M1, M2, and L proteins are considered as potential antigens, whereas G protein is responsible for induction of virus neutralizing antibodies and protection of animals against challenge virus strain. It also contains determinants of virulence and is responsible for the specific serological reactions of the virus. The epitope site III of the rabies virus glycoprotein appears to be a major antigen responsible for protection. Antigenic difference among strains of rabies virus can be easily detected using a panel of monoclonal antibodies directed against the G protein of the virion. The N protein, on the *other hand* is a group protein which cross-reacts with antisera produced from the rabies related viruses. ⁽⁸⁾ Monoclonal antibodies directed against the N protein have been used to differentiate and classify rabies wild isolates into types of lyssaviruses.

Evidence of serological response to rabies virus can be demonstrated by a variety of laboratory techniques, including Mouse Neutralization Test (MNT), Indirect Immunofluorescence Antibody Technique (IIF), Rapid Fluorescent Focus Inhibition Test (RFFIT), Radioimmunoassay (RIA) or Enzyme Linked Immunosorbent Assay (ELISA). Whereas postmortem brain samples are tested using mouse inoculation test, Seller's staining to detect Negri bodies and Fluorescent Antibody Test (FA). The most definitive means of diagnosis of rabies is by virus cultivation from infected tissues using Human diploid cell line (WI-38), Baby Hamster Kidney cells (BHK-21) and chick embryo-related cells (CER), since rabies virus induces minimal cytopathic effect (CPE). Direct fluorescent antibody staining is the only sensitive and rapid test. Mouse Monoclonal Antibodies (MAbs) and Polymerase Chain Reaction (PCR) techniques are also used for the discrimination of rabies virus strains.

Since rabies is almost invariably fatal illness, emphasis has been placed on preventing human infection. Persons likely to be exposed to rabies; for example laboratory workers who handle potentially infected tissues or virus, and veterinarians, should receive pre-exposure prophylaxis. Post-exposure prophylaxis is intended to prevent rabies in all persons who have been

exposed to the virus. Due to adverse side reactions like paralysis, Semple vaccine has been discontinued in most of the developed countries. Tissue culture vaccines are preferred because of their demonstrable safety and efficacy. The vaccine to be administered should have a concentrated minimum potency of 2.5 IU/ml. The proper post-exposure prevention of rabies always included combined vaccination and passive antibody administration. The intended purpose of Human Rabies Immune Globulin (HRIG) and Equine Rabies Immune Globulin (ERIG) is to provide rabies antibody before an active response to the vaccine takes place.⁽⁹⁾

The conventional polyclonal antisera contain a heterogeneous population of antibodies. These vary from animal to animal and batch to batch in the same animal. The sera also contain undesirable non-specific or cross-reacting antibodies and removal of these unwanted antibodies is a time consuming task involving repeated adsorption techniques. The specificities and avidity of these antisera show a lot of variation. The hybridoma technique involving the fusion of myeloma cells with B-lymphocytes specific to a particular antigen overcomes most of the above difficulties. The monoclonal antibodies are homogeneous with a single specificity and affinity. Once a stable clone producing a particular monoclonal antibody (MAb) is established, it provides a continuous and unlimited supply of highly specific and pure antibodies.⁽¹⁰⁾

In 1975, Kohler and Milstein reported successful production of MAbs using hybridoma technique. Nearly 25 years after this discovery, MAbs continue to evolve from remarkably useful laboratory reagents into potent tools for the diagnosis and treatment of various diseases.⁽¹¹⁾

In 1978, Wiktor and Koprowski first described monoclonal antibodies directed against the rabies virus. MAbs were produced by hybridoma of fused mouse myeloma cells and splenocytes from mice immunized with either rabies virus or rabies related viruses. These hybridoma-secreted MAbs were directed against G or N protein of rabies virus. These antibodies displayed specific reactivity patterns which were used to characterize and classify rabies and rabies related viruses into groups corresponding to antigenic determination⁽¹²⁾ Also vaccine strains of rabies were differentiated from wild isolates. Since

then, other hybridomas have been produced and different panels of MAb have been established to allow differentiation of rabies virus isolates from terrestrial and bat host species in the USA, Western Europe and to a lesser extent Africa, Asia, Eastern Europe and Latin America. Nowadays MAb directed to the surface glycoprotein, nucleoprotein, nominal phosphoprotein and matrix protein are used to characterize rabies viruses. The different strains of rabies viruses show marked difference in their reactivity with hybridoma antibodies. Previous application of traditional epidemiological methods to such outbreaks was limited since rabies viruses were considered antigenically similar regardless of their geographic or specific origin. However the availability of MAbs which distinguish closely related natural rabies variants can provide potential clues to the origins of epizootics. Also these can be used to detect the cause of vaccine failure cases.

The greater role for rabies MAbs may prove to be in the field of vaccination or post-exposure treatment for humans using chimeric (Murine-human) or genetically engineered MAbs. More than 10 million people annually receive rabies virus prophylaxis in the form of ERIG or HRIG, together with rabies vaccine. ⁽¹³⁾ However, the world shortage of these immunoglobulins, as well as the risk of both adverse reactions associated with ERIG and the high cost of HRIG, has hampered global efforts to provide a post-exposure prophylaxis against rabies. ⁽¹⁴⁾ For these reasons, it is desirable to develop MAbs to replace HRIG in the rabies virus post-exposure treatment.

The present study was embarked upon keeping this purpose as the focus point. In this study, we aimed to produce anti-glycoprotein and anti-ribonucleoprotein MAbs and determine the different reactivity pattern of selected fixed and street rabies strains to the specific MAbs using IIF technique. Anti rabies MAbs can be used in diagnosis as well as to differentiate rabies strains. However the one main disadvantage of these murine antibodies for therapeutic use in humans is the probable side effect such as anaphylactic reactions and hypersensitization.

Aims and Objectives

- Cultivation of following rabies strains.
 3. Fixed strains: Paris strain, Pitman-Moore (PM), Flury High Egg Passage (HEP), Flury Low Egg Passage (LEP).
 4. Street strains: Street I, Street II
- Maintenance and titration of Challenge Virus Standard (CVS) strain.
- Purification of Ribonucleoprotein (RNP) of rabies virus.
- Production of Anti-rabies monoclonal antibodies (MAbs).
- Purification of MAbs.
- Determination of total protein concentration of MAbs.
- Application of raised MAbs for differentiation of following rabies strains using Indirect Immunofluorescence assay.
 4. Fixed strains: Paris strain, Pitman-Moore (PM), Flury High Egg Passage (HEP), Flury Low Egg Passage (LEP).
 5. Street strains: Street I, Street II
 6. Standard challenge virus: CVS.

Rationale

A few unexplained vaccine failures reported till date, may be due to the inadequacy of the antibodies to neutralize the infecting virus. Also the host system might be non-responsive to the vaccine, or a rabies related virus might be the cause of infection. In some cases, due to antigenic difference between Street and Vaccine strains of rabies, vaccine failure has been occurred. So it becomes necessary to find the antigenic difference between Vaccine and Street strains of rabies viruses. This is possible by using the monoclonal antibodies.

The diagnosis of rabies infection in humans and animals is carried out using antisera. These antisera do not permit to distinguish infections caused by vaccine or street strains of rabies viruses. As per WHO expert committee on rabies, indirect immunofluorescence assay (IIF) is more specific, sensitive and rapid diagnostic test. Due to the presence of common antigens, the use of polyclonal sera got limitations to differentiate virus variants. However, using MAbs produced against rabies viruses can differentiate between these variants. In addition, for epidemiological evaluations these MAbs are an important tool to investigate the prevalence of rabies viruses by using FA test directly on brain smears. Also serum samples can be tested for the presence of antirabies antibodies by using IIF test.

Due permission was obtained from the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), before the start of the project.

Materials

1. Rabies virus strains used in the study

- Standard challenge virus: CVS was obtained from the Department of Virology, Haffkine Institute. This strain was cultivated in primary baby hamster kidney cell cultures. Also it was maintained by intracerebral inoculation in Swiss albino mice.
- Fixed strains: Paris strain, Pitman-Moore (PM), Flury High Egg Passage (HEP), Flury Low Egg Passage (LEP), were obtained from the Department of Virology, Haffkine Institute. These strains were maintained as mouse brain suspension in the vapor phase of liquid nitrogen.
- Street strains: Street I, Street II were isolated from post-mortem human brain samples and obtained from Bombay Veterinary College, Parel Mumbai.

2. Titration of Challenge Virus Standard (CVS) strain

Mice: Swiss Albino mice weighing 12-14 gm (Kasauli strain) were procured from Haffkine Biopharmaceutical Corporation Ltd. Mumbai and they were fed and watered ad libitum and maintained with proper ventilation.

3. Purification of rabies virus ribonucleoprotein

Caesium chloride (CsCl) (Sigma Chemicals)
Octyl- β -D-glucopyranoside (OGP, Sigma Chemicals).

4. Cell lines

Baby Hamster Kidney (BHK-21) cell line
SP2/0 cell line

These cell lines were obtained from National Centre for Cell Science (NCCS), Pune.

5. Media

- Modified Eagles base Medium (MEM) was used to cultivate BHK-21 cell line.
- Dulbecco's Modified Eagles Medium (DMEM) was used to maintain SP2/0 cell line.
- Fetal calf serum.

6. Preparation of MAbs

- Mice: BALB/c mice were procured from ACTREC, Kharghar.
- Media and reagents:
 - Complete Freund's adjuvant (Sigma Chemicals)
 - Rabipur vaccine (Aventis Pharma Ltd)
 - Polyethylene glycol 4000 (PEG, Sigma Chemicals)
 - DMEM + HAT medium (Sigma chemicals)
 - Horse serum

7. Screening of MAbs

- (a) Enzyme Immuno assay: Platelia Rage, Bio-Rad Kit.
- (b) Indirect Immunofluorescent Antibody test:
 - Anti-mouse immunoglobulin FITC conjugate (Sigma)

8. Purification of MAbs

Ammonium Sulphate $[(\text{NH}_4)_2\text{SO}_4]$

Methods

1. Titration of Challenge Virus Standard (CVS) strain

CVS strain was cultivated by mice brain passage. LD₅₀ of the virus was determined by WHO method.

2. *In-vitro* cultivation and purification of rabies virus.

***2.1 In-vitro* cultivation of rabies virus**

BHK-21 cells were grown and maintained in Roux-flasks (1L capacity) in the presence of 80-100 ml Modified Eagles base Medium (MEM) with

10 % Fetal calf serum (FCS).

The rabies virus challenge virus standard (CVS) was adapted and propagated on BHK-21 cells. The cells were infected with rabies virus in Roux bottles having capacity of 1 Lt and incubated for 1 hour at 37°C. The supernatant was discarded and the monolayer was rinsed with five ml of virus medium (MEM without FCS). The virus medium was then replaced with a fresh medium. The Roux bottles were sealed and incubated at 37°C for 60-72 hours in a humidified CO₂ incubator (3-5% Carbon dioxide). Cultures from the 20th passage were pooled to give a total volume of 800 ml. These pooled suspensions were then centrifuged at around 4000g by using cooling centrifuge (Sorvall make) for 10 minutes. The supernatant containing virus was then stored at -70°C till further use. ⁽¹⁵⁾

2.2 Purification of rabies virus ribonucleoprotein (RNP)

BHK-21 cells infected with the CVS virus were scraped. After washing once in PBS (Phosphate Buffered Saline pH 7.2), approximately 2 ml of cell pellet was suspended in 5 ml of ice-cold Distilled water (D/W) containing aprotinin (Sigma). The cells were homogenized in a glass homogenizer and centrifuged for 20 minutes at 1000g. The supernatant was collected. The pellet containing the cell debris was suspended as described above and centrifuged for 20 minutes at 1000g. The

supernatant was collected and the centrifugation step was repeated. The supernatant from Steps 1 and 2 was combined and centrifuged for 10 minutes at 12,000g. The supernatant was collected. Six grams of caesium chloride (CsCl) was added to 15 ml of resulting supernatant. CsCl was dissolved, mixed well and centrifuged for 18 hours at 1,50,000g at 4°C in a SW501 rotor. After centrifugation, the RNP band was collected using a 23-gauge needle, at a buoyant density of 1.31g/cm³. Four grams of CsCl were dissolved in 9 ml of NTE (0.1 mol/l NaCl, 0.01 mol/l Tris-HCl, 0.001 mol/l EDTA, pH 7.5) buffer containing 2% OGP (octyl-β-D-glucopyranoside, Sigma Chemicals). Four volumes of this solution were mixed with 1 volume of RNP collected in Step 4 and were centrifuged for 18 hours at 1,50,000g at 4°C. The band was collected as described in Step 4 and dialysed against PBS (pH 7.2) using dialysis bags (Hi Media) with a 10,000 Dalton cut-off. Six Swiss albino mice were inoculated intracerebrally to check the infectivity of the protein. The purification of the RNP was confirmed by serologic analysis using fluorescent antibody test.⁽¹⁶⁾

3. Preparation of anti-rabies monoclonal antibodies.

3.1 Immunization of mice

Ten to twelve week old female BALB/c mice were immunized by an intraperitoneal inoculation of purified RNP (10µg) with complete Freund's adjuvant (Sigma Chemicals). Other group of mice was immunized by Rabipur vaccine (1 dose/ mouse, potency: 2.5 IU, Aventis Pharma Ltd.). A booster inoculation was given intravenously 3-4 weeks later. Spleens were removed 3-5 days later.⁽¹⁷⁾

3.2 Culturing of mouse Myeloma (SP2/0) cell line

Myeloma Cell line, SP2/0 was maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10 ml of 100-mmol/l sodium pyruvate and 10% fetal calf serum in a humid 5% carbon dioxide incubator. One week before fusion, the cells were transferred to DMEM containing 10% horse serum to improve the fusion

efficiency. The cells were maintained in stationary suspension culture at concentration of 1×10^6 cells/ml and 5×10^6 cells/ml for fusion respectively. All sera were heated at 56°C for 30 minutes to inactivate the complement. ⁽¹⁷⁾

3.3 Production of hybrid cells (Fusion)

Splenocytes from immunized mice were fused with SP2/0 cells using Polyethylene glycol 4000 (PEG, Sigma Chemicals). After fusion, the cells were seeded in 24 wells tissue culture plates in hypoxanthine-aminopterin-thymidine (DMEM-HAT-10FCS) medium (Sigma chemicals). All the plates were incubated at 37°C in 5% CO_2 incubator. The supernatants of the wells with growing hybrids were screened on 16th day of fusion. Enzyme Immunoassay was performed for anti-glycoprotein MAbs and Indirect immunofluorescent antibody test for anti-ribonucleoprotein MAbs production. ⁽¹⁷⁾

3.4 Screening of hybridoma supernatants.

- Enzyme Immunoassay (EIA) :

EIA was performed on ready to use purified glycoprotein (G) coated plates (Platelia Rage, Bio-Rad). A microtitration plate was pre-coated with glycoprotein G. The plate was washed with Tris NaCl buffer (pH 7.4) with 1% Tween 20. Supernatant samples and controls were diluted 1/100 using concentrated skim milk as a diluent. Hundred microliters of each dilution was dispensed per well and the plate was incubated for 60 minutes at 40°C in a humid incubator with 5% CO_2 . The plate was emptied and washed 3 times with wash buffer. Then 100 μl of conjugate (staphylococcus aureus protein A labeled with Peroxidase) was added in each well and the plate was incubated for 1 hour at 40°C in a humid incubator with 5% CO_2 . The plate was emptied and washed 4 times with wash buffer. Hundred microliters of chromogen Ortho-Phenylene Diamine- 2HCl was added to each well and the plate was incubated for 30 minutes at room temperature in the dark. Fifty

microliters of 4 N sulphuric acid was added as a stopping solution. The absorbance was read in a spectrophotometer at 492 nm.

- Indirect immunofluorescent antibody test (IIF):

The virus infected BHK-21 cell monolayer was trypsinized and resuspended in MEM without serum to get approximately 10^4 cells/ml. Ten microliters of the cell suspension were added to each of the 12 well Teflon coated multispot slides. After air drying, the smears were fixed with chilled acetone at -20°C for 30 minutes and washed thrice in PBS pH 7.4-7.6. After air drying, 10 μ l of the hybridoma culture supernatant were added to a well and slide incubated at 37°C for 30 minutes in a moist chamber. At the end of incubation, 3 washings, each for 10 minutes, were given using PBS pH 7.4-7.6. Anti-mouse immunoglobulin FITC conjugate (Sigma) was added to each well in 1:100 dilution and incubated at 37°C for 10 minutes in a moist chamber. The slides were washed again thrice for 10 minutes with PBS. Slides were then mounted in 50% buffered glycerol and observed under fluorescence microscope. ⁽¹⁸⁾

3.5 Cloning of hybridoma.

Positive hybridomas were cloned by limiting dilution method using cloning medium (One part of myeloma culture supernatant and 2 parts of DMEM-20 FCS). After 10-15 days positive clones were selected using screening assays described earlier. ⁽¹⁷⁾

3.6 Bulk production of MAbs.

- ❖ The monoclonal antibodies were produced by injection of hybridoma cells into the peritoneal cavity of syngenic mice. Approximately, 2×10^6 hybridoma cells in 0.5 ml of sterile saline buffer were injected into the peritoneal cavity of 12-20 week old syngenic mice. After 12-15 days the peritoneal cavities were tapped and the mice were sacrificed. The ascites were collected and centrifuged at 200g for 10 minutes to obtain the supernatant.

- ❖ Hybridoma cells were inoculated into cell cultures at a concentration of $2-5 \times 10^6$ cells/ ml. after 6 days, the supernatants were centrifuged at 200 g for 10 minutes, tested for desired antibody production and frozen at -20°C . ⁽¹⁷⁾

4. Purification of MAbs using Ammonium sulphate precipitation.

Forty percent saturated solution of $(\text{NH}_4)_2\text{SO}_4$ was prepared for the precipitation of MAbs. Twenty milliliters of medium containing MAbs were mixed with 20 ml of saturated solution of $(\text{NH}_4)_2\text{SO}_4$ by stirring at $4^\circ - 8^\circ \text{C}$ for 30 min. This was then centrifuged at 5000g for 30 minutes. The pellet obtained was then dissolved in 40 ml D/W. The resulting solution containing MAbs was subjected to dialysis against PBS pH 7.2 using dialysis bags with a 10,000 Dalton cut-off. ⁽¹⁹⁾

5. Determination of the specificity of the monoclonal antibodies.

Indirect immunofluorescent antibody test:

The specificity of anti-ribonucleoprotein MAbs (Anti-RNP) and anti-glycoprotein MAbs (Anti-G) was carried out on rabies infected BHK-21 cells and on infected brain smears. The method is described in 3.4. For this test, the following rabies virus strains were used.

- Fixed strains: Paris strain, Pitman-Moore (PM), Flury High Egg Passage (HEP), Flury Low Egg Passage (LEP) and Challenge Virus Standard (CVS) strain.
- Street strains: Street I, Street II

All the samples were tested twice against MAbs produced to confirm the results

RESULTS

1. Purification of rabies virus ribonucleoprotein (RNP)

Purified RNP was isolated from BHK-21 cells infected with RV-CVS. After centrifugation, the RNP band was collected using a 23-gauge needle, at a buoyant density of 1.31g/cm^3 . The concentration of the protein after dialysis was found to be 0.7 mg/ml . The protein estimation was carried out by the method of Lowry et. al, by keeping bovine serum albumin as a reference. ⁽²⁰⁾ The purity of RNP was confirmed by SDS-PAGE. This purified protein was used as one of the antigens in the study.

2. Production and Screening of hybridoma cells

After 15 days of fusion, the surviving colonies in the selective medium (DMEM-10-HAT) were screened. To select the clones producing Anti-ribonucleoprotein MAbs, we used Indirect Immunofluorescent Antibody Test (IIF) and the selection of clones producing antiglycoprotein MAbs was done by Enzyme Immunoassay (EIA).

- Anti-ribonucleoprotein MAbs (Anti-RNP MAbs): Respective supernatants from all the wells containing surviving colonies were subjected to screening by IIF. The clones giving brightest intracytoplasmic fluorescence characteristic of the antibody to RNP antigen of rabies virus were selected for characterization and specificity study. Out of 42 surviving colonies, 20 colonies were positive. Five nucleoprotein specific colonies were selected, since these colonies were cloned twice by limiting dilution method and were found stable.
- Anti-glycoprotein MAbs (Anti-G MAbs): Supernatants from the wells containing colonies were screened by EIA. A specific absorbance at 492 nm equal to or greater than 0.1 was considered positive for antiglycoprotein antirabies antibody. ⁽²¹⁾ Clones having $>2.0\text{ EU/ml}$ concentrations of antibodies were selected for the study. These clones were subsequently cloned twice. Out of 52 surviving colonies, 30

colonies were found to be positive. From these colonies, 6 colonies were found to be stable after cloning and were selected for the study.

Table 1: Screening results of anti-G MAbs by EIA

Clone	Absorbance at 492nm	Antibody titer (EU/ml)*
Positive control	1.210	7.7
A₁-1	0.458	2.6
A₅-1	0.431	2.4
A₆-1	0.479	2.8
C₃-1	0.422	2.3
C₃-3	0.523	3.0
D₄-3	0.448	2.5

*1 EU/ml $\approx$ 1 IU/ml

(Anti-rabies antibody titer was calculated as per the pack- insert in the Platelia kit- Bio-Rad.)

3. Cloning of hybridoma

After screening, the hybridoma secreting relevant antibodies were expanded in a second 24-well plate. These hybridoma were cloned twice to confirm that they are monoclonal.

4. Purification of Anti-RNP and Anti-G MAbs

The prepared MAbs were purified by Ammonium Sulphate precipitation method and were subjected to dialysis as described above. The protein estimation was carried out by the method of Lowry et. al, by keeping bovine serum albumin as a reference. ⁽²⁰⁾ The protein concentration of all the MAbs was found to be more than 1.0 mg/ ml.

5. Determination of the specificity of the monoclonal antibodies

MAbs directed against ribonucleocapsid can be distinguished from those directed against glycoprotein according to the localization of rabies

antigens in infected cells. Anti-RNP MAbs showed fluorescence in the cytoplasm whereas Anti-G MAbs showed membrane fluorescence. Based on the presence of fluorescence, reactions were classified as positive or negative. Some weak reactions were observed, which were mostly considered as either negative or tested again with a different dilution of the respective MAbs.

Table 2: Immunofluorescence reaction of 5 anti-RNP MAbs with rabies virus strains

	CVS	PARIS	PM	HEP	LEP	Street I*	Street II*
A₂-2	+	+	+	-	+	+	+
B₂-2	+	+	+	+	+	+	+
B₃-2	+	+	+	+	+	+	-
B₆-2	+	+	+	-	+	+	-
C₃-2	+	+	+	+	+	+	+
Anti-N	+	+	+	+	+	+	+

+ = positive reaction

- = no reaction

* = street rabies samples from field isolates

Anti-N = FITC labeled antinucleocapsid Antirabies antibodies

Table 3: Immunofluorescence reaction of 6 anti-G MAbs with rabies virus strains

	CVS	PARIS	PM	HEP	LEP	Street I*	Street II*
A₁-1	+	+	+	-	+	-	+
A₅-1	+	+	+	+	+	+	+
A₆-1	+	+	+	+	+	+	+
C₃-1	+	+	+	+	+	-	-
C₃-3	+	+	+	+	+	-	+
D₄-3	+	+	+	+	+	-	+
HRIG	+	+	+	+	+	+	+

+ = positive reaction

- = no reaction

* = street rabies samples from field isolates

HRIG = Human Rabies Immune Globulin

DISCUSSION

Differentiating rabies virus strains was not possible until the pioneering work of Wiktor and Koprowski in 1978 and the production of first anti-rabies monoclonal antibodies (MAbs). Although the primary rationale for their development was to investigate the role of antigenic difference in vaccine failure, thereafter, it gradually became clear that MAbs could be used to distinguish fixed rabies strains from street strains. The present study was aimed at producing anti-nucleocapsid and anti-glycoprotein MAbs and checking their specific reactivities against Vaccine (Standard) and Street strains of rabies. Four fixed strains [Paris strain, Pitman-Moore, Flury High Egg Passage (HEP), Flury Low Egg Passage (LEP)], one challenge strain (CVS) and two Street strains (I and II) were used to check the specific reactivity against MAbs produced by us. Flury HEP and Flury LEP strains were originated from North America field strains and CVS, Paris strains were originated from Pasteur laboratory strains

All the rabies strains used in the study were cultivated and preserved in the vapor phase of liquid nitrogen. All these strains were inoculated in suckling mice brain as well as in BHK-21 cell line, before the use in the Immunofluorescence assay. These brain tissue samples and infected BHK-21 cells were used for the assay to check the reactivities of the MAbs produced.

We have cultivated the Challenge Virus Standard (CVS) strain *in-vivo*, by using Swiss albino mice and *in-vitro*, in BHK-21 cell line. Rabies virus was found to be released in the BHK-21 cell line supernatant, after 20 reinfections. The LD₅₀ of the virus was found to be 10^{4.3} LD₅₀/ 0.03 ml.

The ribonucleoprotein (RNP) of CVS was isolated and purified by using density gradient centrifugation technique, on a laboratory scale. This purified RNP was subjected to dialysis to remove excess of salts and impurities of low molecular weight. After purification, the RNP was injected intracerebrally into Swiss albino mice to check the activity of the virus. All mice withstood the purified RNP without infection. The result revealed the safety of the antigen for immunization. Also the RNP obtained was checked by

immunofluorescence for its purity. Thus the RNP was proved to be free of virus membrane material and cellular contaminants. This was one of the antigens used for the immunization in hybridoma technology,

In this study, we produced MAbs using hybridoma technology by the fusion of mouse myeloma cells (SP2/0) and B cells from mice immunized with either purified ribonucleoprotein (RNP) or glycoprotein (G). Total 11 MAbs were produced of which 5 were anti-ribonucleoprotein (anti-RNP) MAbs and 6 were anti-glycoprotein (anti-G) MAbs.

Wiktor and Koprowski (1980) used concentrated, purified; β -propiolactone inactivated vaccine prepared from the ERA, CVS, Kelev or Mokola strains. ⁽²²⁾ We have used two purified antigens, RNP and G protein for immunization purpose. The aim was to obtain homologous antibodies against these antigens, so that screening and selection of positive clones becomes easier. RNP protein was obtained by purification of CVS virus, whereas, Rabipur vaccine was used as the G protein source. Both of these antigens were injected along with the complete Freund's adjuvant.

After fusion, on 16th day, the supernatants from the surviving hybridoma colonies were screened by Indirect Immunofluorescence Assay and Enzyme Immuno Assay. For screening, Wiktor and Koprowski used Radioimmunoassay (RIA) ⁽²²⁾

Whereas Indirect Immunofluorescence Assay was used by Kapil et al. ⁽²³⁾ These screening techniques are very simple and easy to perform and also large numbers of supernatants can be screened in short term.

Cloning was an extremely important step, since the hybrids have a tendency to loose chromosomes randomly till they stabilize. Hybrids may become non-secretors during sub cloning due to loss of chromosomes. It is necessary to find all selected clones were stable after two consecutive limiting dilutions. In this study, clones which gave positive results in screening assay were selected. During all these steps care was taken to avoid mycoplasma infection to the cultures. Hybridomas suspecting contamination were rejected from the study.

Large amounts of monoclonal antibodies have been produced by methods involving (a.) Production of ascitic fluid obtained by injecting hybridoma into peritoneal cavity of syngenic mice, and (b.) Production of large volumes of hybridoma supernatants from cell cultures. We have followed both the methods. However, due to the production of non-relevant antibodies present in the peritoneal fluid, which interfere in purification procedure, the cell culture technique was followed. All the hybridoma supernatants were separated and subjected for the purification.

Ammonium Sulphate precipitation was carried out at 4°C to avoid possible chances of bacterial contamination, for purification of MAbs. The advantage of this method is that it is simple, removes unwanted proteins maximally and gives high recovery percentage in a single step. In spite of yielding a low purity product it is still used as a primary or supportive method for the purification of proteins.

Schumacher et al. had purified MAbs by using HPLC ion exchange chromatography. ⁽²⁴⁾ In our study the total protein concentration of the purified MAbs was found to be more than 1.0 mg/ml.

Finally the specificity of MAbs to various rabies viruses was checked using Indirect Immunofluorescence Assay. Each virus sample was processed with the MAbs panel at least twice in different batches to determine the reproducibility of the test. Samples showing weak positive reaction were processed in duplicate with different dilutions of respective MAbs. Acetone fixed smears of BHK-21 cells containing virus as well as non-fixed cells were used for IIF assay. In this study we have found that IIF assay can be done directly on brain samples preserved at -70°C. The technique was found to be very simple to perform and cost effective.

Anti-RNP MAbs: As per table 2, MAbs secreted by hybridomas and B₂-2 and C₃-2 show similar specificities for all selected virus strains. But the epitopes recognized by the clones A₂-2 and B₆-2 are not present on Flury-HEP strain. In addition, the clone B₃-2 and B₆-2 did not neutralize the epitopes on street-II strain. Here the surprising fact is that the MAbs A₂-2 and B₆-2 did not react

with nucleocapsid of the Flury-HEP virus despite the fact that the HEP virus is a derivative of Flury-LEP. Thus, these results reveal that in the IIF assay anti-RNP A₂-2 and B₆-2 allows us to distinguish between Flury-HEP and the other rabies strains maintained in the laboratory. Also street II strain can be differentiated by using B₃-2 and B₆-2 MAbs.

Anti-G MAbs: As per table 3, Hybridoma A₅-1 and A₆-1 showed specificity to all selected strains. However, A₁-1 does not neutralize HEP virus as well as street-I strain. Similarly MAbs produced from clones C₃-1, C₃-3 and D₄-3 do not recognize street-I strain. Also the epitope on Street-II strain was not recognized by clone C₃-1. Thus anti-G MAbs A₁-1, C₃-1, C₃-3 and D₄-3 can be used as a diagnostic tool to differentiate rabies strains. Since four clones in the study failed to neutralize the epitopes on street strains, which might be one of the reasons of vaccine failure.

MAbs produced by hybridomas expressed different specificities in interaction with various strains of rabies virus. A₂-2, B₃-2, B₆-2, A₁-1, C₃-1, C₃-3 and D₄-3 panel of hybridoma antibodies not only provides a tool for the diagnosis of rabies in the tissue of infected subjects but also permits spot viral antigen classification. Also this panel can be useful in investigating origins of viruses involved in post vaccine failures.

According to the work of Wright et al, Antigenic differences in nucleocapsid protein divide the vaccine strains into subgroups. The vaccine strains ERA, SAD and LEP, HEP, can be distinguish from each other and from the strains CVS, Pasture virus and PM. Also anti-glycoprotein MAbs distinguished Pasteur vaccine strain from other strains. ⁽¹⁸⁾ Wiktor and Flamand showed the antigenic difference of RNP between selected rabies strains and HEP strain. Also their work shows in FA test, MAb 377-7 stains nucleocapsids of all fixed and street strains studied except CVS. ⁽²⁵⁾

Above results show that fixed rabies virus falls into antigenic groups in relation to its cross reactivity with antibodies produced by a given hybridoma. Since there are marked antigenic differences among the street strains and vaccine strains; there might be possibility of vaccine failure. This panel can be

used to study the specificity as well as for the discrimination of rabies virus isolated from different hosts in India. Since the MAbs produced by the method described earlier yielded antibodies more than 2 E.U./ml in Enzyme Immuno Assay, the protective study in mice can be done by using these antirabies MAbs against rabies viruses.

According to Schumacher et al, the cocktail of MAbs, consisting of anti-G MAbs and anti-RNP MAbs is highly effective in post exposure treatment. ⁽²⁴⁾ Such a cocktail has several advantages over hyperimmune sera, these MAbs can be used for local wound treatment. For the foreseeable future however there can be no doubt that MAbs will be an essential tool in identifying those factors that affect the maintenance and spread of rabies in nature.

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