Laboratory techniques in rabies

FOURTH EDITION

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Preface

During the 20 years that have elapsed since the publication of the third edition of *Laboratory techniques in rabies*, enormous progress has been made in improving methods of rabies vaccine and antisera production, and in developing new diagnostic and assay procedures. Major advances in molecular biology techniques have been extensively applied to the study of the rabies virus during recent years, and a fourth edition of the monograph has therefore become necessary. This edition includes some 30 new chapters, which describe new diagnostic, research and vaccine production techniques. Although some of these methods are currently restricted to relatively advanced laboratories (e.g. monoclonal antibody techniques, the polymerase chain reaction and virus expression systems), they are expected to become routine procedures in the future. Nevertheless, many laboratories will not have the facilities or equipment to use these methods, therefore the basic classical techniques described in the previous edition have been retained and, where necessary, brought up to date.

The production of rabies vaccines for animal and human use is extensively reviewed. The production of modified live-virus vaccines and recombinant vaccines is also briefly covered. It should be noted that there has been a dramatic increase in the number of cell-culture vaccines available for human use and that production is no longer restricted to developed countries. Many of these vaccines have now replaced those derived from nerve tissue. Accordingly, only two chapters deal with the production of the latter, which are still used in some developing countries.

It should be stressed that claims for the efficacy of particular vaccines are entirely the responsibility of the authors, and that their inclusion in this book does not imply official recognition by WHO. Vaccine manufacturers intending to use the production techniques described here should refer to the requirements for rabies vaccines for human and veterinary use, as defined by the WHO Expert Committee on Biological Standardization.[1-3]

An early draft manuscript of this fourth edition of *Laboratory techniques in rabies* was examined by the WHO Expert Committee on Rabies in September 1991,[4] and a number of suggestions were made for changes to the text and for the

[1] *WHO Expert Committee on Biological Standardization. Thirty-first report.* Geneva, World Health Organization, 1981 (WHO Technical Report Series. No. 658), Annex 2; Annex 3.

[2] *WHO Expert Committee on Biological Standardization. Thirty-seventh report.* Geneva, World Health Organization, 1987 (WHO Technical Report Series, No. 760), Annex 9.

[3] *WHO Expert Committee on Biological Standardization. Forty-third report.* Geneva, World Health Organization, 1994 (WHO Technical Report Series, No. 840), Annex 4; Annex 5; Annex 6.

[4] *WHO Expert Committee on Rabies. Eighth report.* Geneva, World Health Organization, 1992 (WHO Technical Report Series, No. 824).

inclusion of some additional material. In preparing the final manuscript, the editors have ensured that the information is as up to date as possible. Where new material could not be incorporated in the existing text, it has been added in the form of appendices at the end of the book.

The World Health Organization gratefully acknowledges the collaboration of the many eminent specialists who have contributed to this volume. The editors thank Miss C. Allsopp, Office of Publications, WHO, for her assistance in the preparation of this book.

List of acronyms and abbreviations used in this book

ABT	antibody-binding test
ATCC	American Type Culture Collection
BHK	baby hamster kidney cells
BPL	beta-propiolactone
BSA	bovine serum albumin
CDC	Centers for Disease Control and Prevention (USA)
cDNA	complementary deoxyribonucleic acid
C-ELISA	competitive enzyme-linked immunosorbent assay
CER	chick embryo-related cells
CI_{95}	95% confidence interval
CVS	Challenge Virus Standard
DI	defective interfering (particles)
DIA	dot-immunobinding assay
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
EAE	experimental allergic encephalomyelitis
EBL	European bat lyssavirus
EBM	Eagle's basal medium
ED_{50}	median effective dose, 50% end-point dilution
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
EMEM	Eagle's minimum essential medium
ERA	Evelyn Rokitniki Abelseth strain of rabies virus
ERIG	equine rabies immunoglobulin
FA	fluorescent antibody
FCS	fetal calf serum
FDA	Food and Drug Administration (USA)
FFD_{50}	dilution at which 50% of the observed microscopic fields contain one or more foci of infected cells
FFU	focus-forming units
FITC	fluorescein isothiocyanate
FRhMDC	fetal rhesus monkey diploid cell
FWR	French wild rabies isolates
G protein	rabies glycoprotein
HDC	human diploid cell
HEP	Flury high egg passage strain of rabies virus
HN	haemagglutinin–neuraminidase protein
HRIG	human rabies immunoglobulin

Ig	immunoglobulin
INHV	infectious haematopoietic necrosis virus
INPPAZ	PAHO/WHO Pan American Institute for Food Protection and Zoonoses (Argentina)
IU	International Unit
LD_{50}	median lethal dose
LEP	Flury low egg passage strain of rabies virus
L protein	rabies RNA-dependent RNA polymerase
M1 protein	rabies phosphoprotein
M2 protein	rabies matrix or membrane protein
MAb	monoclonal antibody
MAb-G	anti-glycoprotein monoclonal antibody
MAb-N	anti-nucleoprotein monoclonal antibody
MAb-RNP	anti-ribonucleoprotein monoclonal antibody
MEM	minimum essential medium
$MICLD_{50}$	median lethal dose for mice inoculated by the intracerebral route
MIT	mouse inoculation test
MLV	modified live-virus
MNA	mouse neuroblastoma cells
MNT	mouse neutralization test
MOI	multiplicity of infection
Mok	Mokola virus
mRNA	messenger ribonucleic acid
NA	neuroblastoma cells
NIH	National Institutes of Health (USA)
N protein	rabies nucleoprotein
OD	optical density
PAb	polyclonal antibody
PAb-G	anti-glycoprotein polyclonal antibody
PAHO	Pan American Health Organization
PAS	Louis Pasteur strain of rabies virus
PBS	phosphate-buffered saline
PCEC	purified chick embryo cell
PCR	polymerase chain reaction
PDE	purified duck embryo
PDL	population doubling level
PFU	plaque-forming units
PHKC	primary Syrian hamster kidney cell
PM	Pitman-Moore strain of rabies virus
PSRV	product-specific reference vaccine
PV	Pasteur strain of rabies virus
PVRV	purified Vero cell rabies vaccine
RIG	rabies immunoglobulin
RNA	ribonucleic acid
RNP protein	rabies ribonucleoprotein
RTCIT	rabies tissue-culture infection test
RFFIT	rapid fluorescent focus inhibition test
RREID	rapid rabies enzyme immunodiagnosis
SAD	Street-Alabama–Dufferin strain of rabies virus

SCID	severe combined immunodeficient
SDS–PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SMB	suckling mouse brain
$TCID_{50}$	median tissue-culture infective dose
VRG	recombinant vaccinia virus expressing the G protein gene of rabies virus
VSV	vesicular stomatitis virus

PART I

General considerations

CHAPTER 1

Safety precautions in handling rabies virus

M. M. Kaplan[1]

Various publications are available describing the precautions to be taken in laboratories working with infective agents. The references listed at the end of this chapter include four manuals (*1–4*) that deal with problems associated with handling such agents, and Chapter 4 and Appendix 1 of this monograph are concerned with the shipment and preparation of specimens from animals suspected of being rabid. The present chapter discusses some properties of the rabies virus and the precautions needed to protect personnel. It is noteworthy that apparently only one human death has occurred from a laboratory infection with fixed virus, despite accidental needle punctures and other exposures to rabies virus during many decades of handling this virus in research, diagnostic and vaccine production laboratories. This death was reported in 1972 in a laboratory worker in Texas, USA, who was engaged in the preparation of vaccine and was exposed to fixed virus in an aerosol from a blender (*5*). Another laboratory infection from an aerosol subsequently occurred in New York state, USA, and the victim was still alive some 10 years later, although with severe residual mental impairment (W.G. Winkler, personal communication). Nevertheless, the fear surrounding work involving rabies virus has resulted in relatively few laboratories conducting needed research in this field. It is hoped that the following discussion will dispel undue concern, and will provide guidance on rational steps to be taken to avoid accidents and on how to deal with them when they do occur.

Properties of the virus

The rabies virus belongs to the genus *Lyssavirus*, family Rhabdoviridae: enveloped and bullet-shaped viruses that contain lipid and single-stranded RNA (see Chapter 3). It is sensitive to lipid solvents (soap solution, ether, chloroform, acetone), 45–70% ethanol, iodine preparations, and quaternary ammonium compounds (*6*). Other relevant properties are resistance to drying, to repeated freezing and thawing, relative stability at pH 5–10, and sensitivity to pasteurization temperatures and ultraviolet light. The virus is readily inactivated by β-propiolactone, but is more resistant to 0.25–0.5% phenol used in Semple-type vaccines, where several days are required to obtain complete inactivation (see Chapter 20).

Pathogenesis

The virus is usually introduced by a bite wound, although penetration can occur through intact mucous membranes and the digestive tract (*7*), but not through

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intact skin. In tissue-culture systems, the virus penetrates into the cell within 15 minutes, after which it can no longer be neutralized by specific antiserum (*8*). Airborne natural infection is possible in exceptional circumstances, for example in caves harbouring large numbers of bats carrying the virus (*9*).

There are marked differences between different strains of virus in their ability to infect, spread within the body, and produce disease. Laboratory strains of "fixed" virus used to produce vaccines, or employed in diagnostic and research procedures, have low pathogenicity when inoculated peripherally in low doses. The Fermi-type vaccine, as well as the original Pasteur vaccine, contained residual, live, fixed virus. Such vaccines were used for many decades, but are no longer recommended by the WHO Expert Committee on Rabies. Accidents occurred in humans when such fixed virus vaccines were not properly incubated to reduce their virus content, and large amounts of material of a high titre were injected into humans (*10*). Laboratory accidents involving small puncture wounds are not very dangerous when fixed or modified attenuated virus is handled. They need not cause undue worry if appropriate wound treatment procedures and a booster vaccine dose are employed (see below). This includes all fixed or modified strains such as SAD (Street-Alabama–Dufferin), ERA (Evelyn Rokitniki Abelseth), CVS (Challenge Virus Standard), LEP (Low Egg Passage) and HEP (High Egg Passage) strains.

Street virus, however, must always be handled with respect, especially in the presence of hyaluronidase-containing saliva, despite apparent differences in the ability of different strains of street virus to infect through peripheral inoculation. This ability to infect and cause disease in animals is mainly a function of virus dosage, i.e. there appears to be a threshold below which disease is not produced. The susceptibility of humans to small amounts of street rabies virus is apparently not as great as that of foxes and cattle, but since human infection has been known to occur even after relatively small puncture wounds (on the fingers for example), it is wise to consider all wounds contaminated with street virus as potentially very dangerous. In any event, all laboratory personnel should receive pre-exposure immunization (see page 6).

Laboratory precautions

All laboratories have their individual arrangements and consequent rules of discipline for handling infective materials (*1–4*). No attempt, therefore, is made here to cover the many possible variations of measures that could be used. Instead, general recommendations are given below for procedures that could be adapted to meet the major laboratory operations employed with rabies virus.

Protective clothing

Gross operations requiring the opening of skulls and spinal column, or those in which splintered material is encountered (broken glassware, bone), should be performed with thick protective gloves, sleeved gowns, and goggles or a plastic face shield. Close-fitting goggles and a face mask are always wise precautions, especially when street virus is handled. Rubber or strong plastic aprons that can easily be disinfected or discarded should also be worn. Close-fitting plastic or

rubber gloves should be worn when animals are being inoculated with street virus; gloves are not necessary, and are often a hindrance, when fixed or other attenuated strains of rabies virus are used in tissue culture and when titrations are performed.

Aerosols

Since airborne rabies infection has been demonstrated (see above), high-speed mixing and centrifugation procedures should be carried out in tightly closed containers and under a negative draught hood. Other operations that might cause aerosols (e.g. pipetting) should also be carried out under a negative draught hood. Hoods and cubicles should be provided with ultraviolet lamps for disinfection when not in use. Pipetting by mouth should be prohibited.

Disinfectants

Quaternary ammonium disinfectants in 1:500 dilution, 45–70% alcohol, 1% soap solution, and 5–7% iodine solutions kill the rabies virus within one minute (*6*) and are indicated for the treatment of wounds (see below). For pipette receptacles a 1:1000 dilution of a quaternary ammonium compound, any iodine disinfectant with residual available iodine of at least 1: 10 000, or a 1% concentration of soapy water or detergent can be used. The solution should be autoclaved and discarded after each use. Hot soapy water or detergent can be used for swabbing floors and tables.

Glassware, plasticware and instruments

These should be discarded into plastic or glass receptacles containing one of the disinfectants mentioned above. They should be autoclaved before reuse or disposal.

Carcasses and animal tissues

These are best disposed of in plastic bags and incinerated. Oral transmission of rabies in laboratory animals may occur when brain tissue containing large amounts of virus is fed (*7*). In countries at an early stage of economic development the carcasses of domesticated animals (sheep and goats) that have been used to prepare Semple-type vaccines are sometimes employed for human consumption— after the head, viscera and vertebral column are removed. Although the risk is thought to be negligible once the meat has been thoroughly cooked, this practice is not recommended.

Treatment of wounds

All wounds should be washed immediately and thoroughly for several minutes with soap and water. This is perhaps the single most important procedure in preventing infection. The washing should be gentle in order to avoid further traumatization of the tissues. All soap should be removed before one of the chemical disinfectants

mentioned under "Laboratory precautions" is applied. Suturing of gaping wounds should be delayed for as long as possible (from several hours up to 3 days). Puncture wounds should be probed gently with an appropriate chemical disinfectant, taking care to minimize further trauma. Local infiltration of wounds with human or animal antirabies immunoglobulin (or serum) should also be employed, if the wounded area so permits; this procedure is used particularly in puncture wounds where adequate cleansing and disinfection are not feasible. Additional measures, such as administration of antimicrobials or antitetanus procedures, when indicated, should follow the local treatment.

Pre-exposure immunization

Various schedules have been tried for pre-exposure immunization of laboratory personnel. The course of action recommended in the eighth report of the WHO Expert Committee on Rabies (1992) should be followed (*11*). The report states:

> Pre-exposure immunization should be offered to persons at high risk of exposure, such as laboratory staff working with rabies virus, veterinarians, animal handlers and wildlife officers, and other individuals who are living in or travelling to areas where rabies is endemic.
>
> Such immunization should preferably consist of three full intramuscular doses of tissue-culture rabies vaccine of potency at least 2.5 IU per dose given on days 0, 7 and 28. (A few days' variation is not important.) The presence of virus-neutralizing antibodies in vaccinated individuals should be ascertained, where feasible, using serum samples collected 1–3 weeks after the last dose. For adults, the vaccine should always be administered in the deltoid area of the arm. For young children, the anterolateral area of the thigh is also acceptable. The gluteal area should never be used for vaccine injections, since administration in this area results in lower neutralizing antibody titres.
>
> Tissue-culture or purified duck-embryo rabies vaccines of potency at least 2.5 IU per dose have been shown to induce adequate antibody titres when carefully administered intradermally in 0.1 ml volumes on days 0, 7 and 28. After reconstitution of the vaccine, the entire volume should be used as soon as possible. Separate syringes and needles must be used for each dose. Intradermal application of the vaccine is of special interest in areas where economic constraints limit vaccine availability. However, pre-exposure vaccination with human diploid cell (HDC) vaccine administered intradermally should, whenever possible, be performed before starting antimalarial prophylaxis, since virus-neutralizing antibody titres have been shown to be lower in patients receiving chloroquine phosphate. When this is not feasible, HDC vaccine should be administered intramuscularly.
>
> Periodic booster injections are recommended for persons at continuing risk of exposure to rabies. The following guidelines are recommended for determining when boosters should be administered:
>
> All persons who work with live rabies virus in a diagnostic, research or vaccine production laboratory should have a serum sample tested for rabies virus-neutralizing antibodies every 6 months and a booster administered when the

titre falls below 0.5 IU/ml. Responsible authorities should ensure that all staff are properly immunized.

All other persons at continuing risk of exposure to rabies should have a serum sample tested for rabies virus-neutralizing antibodies every year; a booster should be administered when the titre falls below 0.5 IU/ml.

Satisfactory antibody responses have been obtained with as little as two or three doses of vaccine given 3 days apart, and this may be tried if there is a shortage of time. A booster dose should be given one to several months after the last inoculation. Any potent tissue-culture vaccine can be employed either intradermally (0.2 ml total dose in two sites in the deltoid region of the upper arm, 0.1 ml in each) or subcutaneously or intramuscularly (in three doses of at least 2.5 IU per dose). Alternatively, 2 ml of 5% nerve-tissue emulsion or its equivalent may be given subcutaneously or intramuscularly if cell-culture vaccine is not available. It is important to determine, as stated above, whether virus-neutralizing antibody has in fact resulted from the procedure. With pre-existing antibody and the known rapid recall response elicited by booster doses, if re-exposure occurs a single inoculation of vaccine can be employed with reasonable confidence. The development of new, concentrated, and purified vaccines of cell-culture origin, with greatly increased antigenic content, has considerably improved the antibody levels now achieved with most conventional antirabies vaccines (*12*).

Exposed individuals who have not received pre-exposure immunization should be treated according to the recommendations made in the eighth report of the WHO Expert Committee on Rabies (*11*), i.e. local treatment of the wound followed by a complete post-exposure course of vaccine, including rabies immunoglobulin if indicated.

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CHAPTER 2

An overview of laboratory techniques in the diagnosis and prevention of rabies and in rabies research

F.-X. Meslin[1] *& M. M. Kaplan*[2]

Introduction

The laboratory occupies a central place in efforts to meet the threat of rabies. Laboratory results influence both the decision whether or not to proceed with a course of treatment, and the decision on the need to institute elaborate measures for controlling an epizootic in a community. The laboratory must also provide the necessary assurance that the biological products used for treatment and prevention in humans and animals are efficient and safe.

The succeeding chapters in this manual describe selected methods for arriving at a diagnosis in the laboratory, for determining the acceptability of biological products in rabies prophylaxis, and for conducting rabies research. Most laboratory workers can decide for themselves whether one or other of the techniques given here is within their competence, but often they are not aware of certain pitfalls and limitations of particular methods. In addition, a choice of procedures can ease the work and provide a decisive answer more quickly. These considerations are partially covered in the relevant sections of this manual; here the various techniques are reviewed and evaluated comparatively to serve as a possible guide for selecting procedures and for interpreting the results obtained. Many of these techniques can be used by laboratories with limited resources.

The institution of treatment measures in exposed individuals, as recommended in the eighth report of the WHO Expert Committee on Rabies (*1*), should never await the results of laboratory diagnosis. A laboratory diagnosis may be delayed for a variety of reasons and early treatment, both local and systemic, can be a critical factor in saving the life of the patient.

A laboratory report should be as clear and unequivocal as possible, and should stipulate exactly the procedures used. A positive test by any one of several recognized procedures overrides negative reactions in the others. Where a doubtful result is obtained in any single test, recourse to the other tests available is essential in order to arrive at a definitive conclusion. Until this conclusion is reached, treatment should be continued. Even with a negative laboratory report, circumstances may occasionally justify the initiation or continuation of treatment by the physician, e.g. suspicious clinical signs in the animal, or an attack in an area

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where rabies is enzootic by an animal that could not be caught or killed. Considerable experience has demonstrated, however, that a complete set of negative results obtained in a reliable diagnostic laboratory can usually be accepted with confidence, and that the treatment can be terminated or modified at that point.

Diagnostic procedures for antigen detection

Fluorescent antibody (FA) test

A quick and easy procedure for the diagnosis of rabies is the use of a suitable dye for the detection of Negri bodies (see Chapter 5). Histopathological techniques have, however, been replaced in most laboratories by the fluorescent antibody (FA) test, which was first developed in 1958 by Goldwasser & Kissling (*2*) and subsequently modified in 1973 by Dean & Abelseth (*3*) and in 1975 by Kissling (*4*). The FA test is now the most widely used method for diagnosing rabies infection in animals and humans. It is based on microscopic examination, under ultraviolet light, of impressions, smears or frozen sections of brain or nervous tissue after treatment with antirabies serum or globulin conjugated with fluorescein isothiocyanate. The test is accurate and results can often be obtained within 30 minutes of receipt of the specimen, although for routine purposes a period of 2–4 hours is desirable for the fixation in cold acetone.

Apart from an appropriate microscope, the two main requirements for success in using this technique are well trained personnel and conjugated antiserum or globulin of good quality. After one year's experience, most laboratories find over 99% agreement between the FA test and the mouse inoculation (MI) test. In the first year, however, some laboratories may miss up to 10% or even 20% of the positives with the FA test and for this reason, both tests (MI and FA) should be run in parallel during this period (*5*). Stringent control of the labelled antirabies antibodies should be carried out to determine the specificity of the fluorescence and to minimize the number of false-positives (*3, 6*). Appropriate tissue sampling is also important. Examination of impressions or smears of tissue samples from Ammon's horn and brain stem are recommended (see Chapter 4). Labelled rabies antibodies can be prepared against the whole rabies virus. Details of the preparation of the immunizing antigen and inoculation schedule in laboratory animals and the separation and labelling of rabies antibodies are given in Appendix 2. More highly potent antisera can be prepared using purified and concentrated rabies virus (*7, 8*) or virion components such as ribonucleoprotein (*9*). Conjugated monoclonal rabies antibodies are being increasingly used in routine diagnosis (*10*). The specificity of these latter conjugates is greater than those prepared against the whole virion or virion components. A conjugate composed of two labelled monoclonal antibodies (502.2 and 103.7) is now widely used and is available commercially. Panels of monoclonal antibodies are also used in studying the epidemiology of rabies (see page 12 and Chapters 11 and 12).

The FA technique is a highly sensitive method for detecting rabies antigen in fresh specimens. However, it may also be performed on fixed specimens (*11–13*). The specimen should be treated with one or more proteolytic enzymes such as trypsin or pepsin before staining to unmask the antigenic sites. The sensitivity of the test using fixed specimens has been reported to be 90–100% of that obtained

using fresh specimens (*13*). However, it is recommended that fresh tissue be examined where possible.

When specimens are received in 50% glycerol–saline, it is imperative that the tissue be washed several times in saline before staining.

Cell-culture isolation techniques

Fixed rabies viruses can grow in a wide variety of cells (*14, 15*). Successful *in vitro* cultivation of rabies virus was first reported in 1936 (*14*). This property has been used extensively in research on rabies (page 15). However, it is only recently that techniques for the isolation of street rabies from suspect material in cell cultures have been developed. Tests for the isolation of street rabies in cell culture were first carried out in the mid-1970s using baby hamster kidney cells, line 21 (BHK-21), and chick embryo-related (CER) and neuroblastoma cells. These studies demonstrated that rabies infection could be detected by immunofluorescence from as early as 4–5 hours up to 5 days following inoculation (*5, 16–19*). Furthermore, it was found that BHK-21 cells were comparable in sensitivity to mice, whereas neuroblastoma cells were more sensitive than mice to infection by street rabies virus. The difference in sensitivity between neuroblastoma cells and BHK-21 cells and other cell lines was reported to be associated with the neural origin of the former (*20, 21*). However, Webster & Casey (*22*) suggested that the difference may also be related to viral strain differences, as well as to cell type.

In routine rabies diagnosis, positive specimens contain amounts of antigen that can easily be detected by the FA test, the MI test or virus isolation in neuroblastoma cells. In the latter case, the result is obtained within 18–24 hours, although rabies antigen may be detected in these cells by FA as early as 4–5 hours after inoculation. Furthermore, virus isolation in cell culture has been shown to be as efficient as the FA test and the MI test for demonstrating small amounts of rabies virus (*20*). However, specimens containing a small amount of rabies virus and which are negative by FA and subsequently positive by virus isolation in cell culture require an incubation period of 4 days after inoculation of the cells.

In view of the usually short delay in obtaining the result, isolation of rabies virus in cell culture should replace intracerebral mouse inoculation whenever possible. It should, however, be borne in mind that only laboratories where cell-culture techniques are currently used can successfully maintain neuroblastoma cells for diagnosis.

Enzyme-linked immunosorbent assay (ELISA)

In the rabies field the enzyme-linked immunosorbent assay (ELISA) was initially developed for the titration of rabies virus-neutralizing antibodies (*23*). The technique was applied to the quantification of rabies antigen by Atanasiu et al. using fluorescein-labelled IgG to the purified nucleocapsid (*24–26*). Subsequently, Perrin et al. (*27*) developed an ELISA called rapid rabies enzyme immunodiagnosis (RREID), which was based upon the detection of rabies virus nucleocapsid antigen in brain tissue. In this test, microplates are coated with purified IgG and an IgG–peroxidase conjugate is used to react with immunocaptured antigen. This technique was compared with the FA test in a collaborative study involving six

laboratories in Europe and North America. The study showed a good correlation between the FA test and RREID, although the latter test was less sensitive (*28*). A further study was organized to evaluate the RREID under conditions prevailing in rabies laboratories in developing countries. The study found over 96% agreement between the FA test and RREID (*29, 30*). Similar results were obtained in a study on more than 3000 specimens (*31*). It should be noted, however, that in view of its lower sensitivity, RREID should not replace FA in laboratories where FA is already performed.

RREID is a simple and relatively cheap technique, which can be especially useful for epidemiological surveys. It may be used to examine partially decomposed tissue specimens for evidence of rabies infection, but it cannot be used with specimens that have been fixed in formalin. Since the antigen can be visualized with the naked eye, the test can be carried out in laboratories that do not have the necessary equipment for FA tests (*32*).

Virus identification using monoclonal antibodies

Monoclonal antibodies are produced by hybridomas of fused mouse myeloma cells and splenocytes from mice immunized with either the rabies virus or rabies-related viruses. They were first produced by Wiktor et al. in 1978 (*10, 33*). These hybridomas secreted monoclonal antibodies directed against the glycoprotein (G protein) or nucleocapsid of rabies virus. Detailed information on the production and use of monoclonal antibodies is given in Chapters 11 and 12. The monoclonal antibodies displayed specific reactivity patterns which were used to characterize and classify rabies and rabies-related viruses into groups corresponding to antigenic determinants. Since then, other hybridomas have been produced and different panels of monoclonal antibodies 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 (*34–42*). Between 1982 and 1990, WHO coordinated collaborative studies on the use of monoclonal antibodies in rabies diagnosis and research (*43–48*). These studies led to the establishment of two panels of monoclonal antibodies, allowing identification of the various lyssavirus serotypes and the differentiation of major virus strains used for vaccine production from field virus isolates. An additional panel of monoclonal antibodies was also selected to differentiate rabies viruses isolated from terrestrial animal species from those isolated from European bat species.

Although monoclonal antibodies are mainly used for epidemiological investigations, they were found to be very useful for rabies diagnosis in certain circumstances, such as imported cases of human rabies and rabies associated with uncertain exposure (*49, 50*), and also routinely in countries where large-scale programmes for oral vaccination of foxes are under way to establish that no infections are caused by the vaccine strain (*41, 51, 52*).

Intra vitam diagnosis

In addition to the brain and spinal cord, rabies virus antigen can be detected by FA in the peripheral nerves, salivary glands, saliva, and also in the cornea and skin

during the final stages of the disease (*53, 54*). *Intra vitam* diagnosis of rabies by FA in corneal impressions was first described by Schneider in animals (*55*) and by Cifuentes et al. in humans (*56*). However, a study of the reliability of corneal impressions for rabies diagnosis showed that, especially when sampling is done under field conditions, a negative result could not rule out the diagnosis of rabies (*57*). Examination of skin biopsy material was shown to be a valuable technique for *intra vitam* diagnosis of rabies in animals and humans (*58–60*). Anderson et al. (*61*) showed that rabies antigen may be detected in skin biopsies from humans at the onset of clinical signs. In contrast, Blenden et al. (*59, 60*) found that only some patients (25–50%) showed positive results during the early phase of clinical illness and that the proportion of positive results increased as the disease progressed.

Specimens for *intra vitam* diagnosis should be of a good quality. They should be refrigerated immediately after collection and until the test is carried out. This is important, since partially autolysed specimens will reduce the percentage of positive results and contamination of the material may lead to false-positive results (*62*). Examination of skin biopsies may also be used for post-mortem diagnosis in countries where opening of the skull of the dead person is not accepted by relatives on cultural or religious grounds.

Tests for the determination of rabies antibody

Reference tests

Serum neutralization assays are used to determine the potency of rabies serum and immunoglobulins for post-exposure treatment, and to evaluate the immunogenicity of human and, to a lesser degree, animal rabies vaccines. The standard procedures recommended at the seventh meeting of the WHO Expert Committee on Rabies (*51*) were the mouse neutralization test (MNT) (*63*) and the plaque reduction assay (*64*). Since then, plaque reduction methods have been superseded by fluorescent focus inhibition tests, which are more convenient. Although the MNT is still widely used as a reference test, the rapid fluorescent focus inhibition test (RFFIT) (*65*) has become the test of choice in most modern laboratories (see Chapter 15). The RFFIT has been shown to be at least as sensitive as the MNT in measuring virus-neutralizing antibodies (*66, 67*), and results have also been shown to correlate well with other tests such as the soluble antigen fluorescent antibody test (*68*), passive haemagglutination (*69*) and radioimmunoassays (*70*). Reported differences (*71–73*) in the potencies of rabies antibody preparations, as measured by the MNT and RFFIT, were not confirmed in a collaborative study carried out under the aegis of WHO (page 15).

A modified version of the RFFIT, called the fluorescent inhibition microtest (FIMT), which uses microtitration plates instead of tissue-culture chamber-slides, has recently been described (*74, 75*). Results of the test have been shown to correlate well with the RFFIT and MNT in measuring virus-neutralizing antibodies.

Techniques under development

Enzyme immunosorbent assays

Enzyme immunosorbent assays for rabies antibody determination were first used during the late 1970s (*23*). Various test systems have since been developed

(*76–80*) for antibody titration in body fluids and for screening supernatants of hybridoma cultures (*77, 80*).

Various adsorbed antigens are used in these systems, including whole virion, purified G protein and purified ribonucleoprotein (RNP) (*81*). An ELISA using purified G protein has been used to determine virus-neutralizing antibody levels in the serum of several species, including humans. The test appears to correlate well with the MNT. In laboratories where mouse colonies are scarce and tissue-culture techniques are unavailable, this test may be considered a suitable alternative to the MNT.

Rapid semi-quantitative assays

Dot-immunobinding assays (DIA) have recently been developed for the diagnosis of rabies based upon the detection of viral antibodies in the serum (*82*). A DIA has been used to determine rabies antibody levels in human sera and the results compared with the RFFIT (*83*). However, the test will need to be slightly modified to enable rabies virus-neutralizing antibody levels to be determined. A DIA using nitrocellulose for antigen support has been used to test for the presence of rabies antibodies in a drop of blood (without centrifugation) (*84*). The test can be carried out in the field and provides results within about 30–40 minutes. It also appears to correlate well with the RFFIT.

A rapid agglutination test (RAT) based upon the ability of specific antibodies to agglutinate sensitized latex beads has recently been developed (*85*). The test can detect post-vaccination antibody levels equal to or higher than 2.5 IU/ml. Above this value, the agreement between this test and the MNT is over 97%. The test is currently being adapted to use whole blood and a mixture with the latex beads on paper instead of a glass slide.

Potency tests

Rabies vaccines

The potency of every vaccine batch must be checked before its release. The appropriate potency tests described in Part V should be used. In spite of disadvantages such as lack of accuracy, variability and poor reproducibility, the NIH test remains the test of choice for inactivated rabies vaccines for both manufacturing and control laboratories (*86–88*) (see Chapter 37).

During the past decade, WHO has coordinated research on potency tests for rabies vaccines (*89–92*). The possibility of replacing the standard NIH test by a modified *in vivo* test was investigated. Various modifications were used—in the challenge strain, number of vaccinations, route of vaccination, and route of challenge (*93*). Some of these modified tests (especially the "peripheral challenge" test developed by the Centers for Disease Control and Prevention) were shown to correlate better with the degree of protection conferred by the vaccine. However, the results of the latter test still showed a high degree of variability and required further modifications for routine use. A number of *in vitro* tests for the measurement of vaccine G protein content were also assessed (*94–102*). A protocol for the replacement of *in vivo* tests by *in vitro* tests was suggested by a WHO Consultation on Rabies in July 1988 (*92*) and was further elaborated during a WHO Consultation on rabies vaccine potency testing in May 1991 (*103*). However, studies showed the

effect of strain differences and substrates for the cultivation of the rabies antigen on the results of the various tests. Some of the *in vitro* tests failed to differentiate between free and virion-bound G protein, although it is now well accepted that only the latter is immunogenic.

In addition, recent studies (*104, 105*) have shown the importance of RNP in the immune response to rabies infection, thereby underlining the need for further studies on the content of RNP in rabies vaccines in relation to that of the G protein and the level of protection.

Rabies immunoglobulins

Until 1984, antirabies sera were calibrated against the International Standard for Antirabies Serum, which was established by the WHO Expert Committee on Biological Standardization in 1955 (*106*). It was a crude hyperimmune horse serum. In 1984, the International Standard for Antirabies Serum, Equine, was replaced by the International Standard for Rabies Immunoglobulin, with a potency of 59 IU per ampoule. The calibration of the human rabies immunoglobulin was made by the RFFIT relative to the equine antirabies serum (*107*).

In 1986, it was reported that some laboratories had obtained significantly different estimates of relative potencies of rabies antibody in human rabies immunoglobulin preparations when these potencies were determined by the MNT and the RFFIT (*71–93*). Results obtained in these laboratories by the RFFIT were consistently lower than those obtained by the MNT. The potential implication in post-exposure treatment was an underestimation of the potency of immunoglobulin preparations with the RFFIT, which could lead to suppression of active rabies immunization. In addition, in some of these laboratories the results of the MNT were influenced by species differences between reference and test preparations. Following these reports, a collaborative study (*108*) was initiated by the International Laboratory for Biological Standards at the State Serum Institute in Copenhagen, Denmark, and its results reviewed at the thirty-ninth meeting of the WHO Expert Committee on Biological Standardization in October 1988 (*109*). The study did not confirm the difference between the two techniques and it was concluded that the International Standard for Rabies Immunoglobulin of human origin should continue to be used to calibrate the other reference materials of human or equine origin required for estimating the potency of preparations containing rabies antibodies. Furthermore, the study showed that the RFFIT was the best technique for evaluating the potency of rabies immunoglobulin and serum preparations. Laboratories using the MNT were consequently advised to verify that their results did not differ from those obtained by the RFFIT. If differences were reported, these laboratories were advised to either switch to the RFFIT or introduce a suitable correcting factor.

Research techniques

Since the early 1980s, molecular biology techniques have been used extensively in studies on the rabies virus. Most of the current research on the development of rabies vaccines, including procedures for their licensing and release, and on the pathogenesis of the virus is based on these techniques.

Cloning and sequencing techniques have led to the determination of the organization of the rabies genome (*110–112*) (see also Chapter 3). The nucleotide sequences of all five genes coding for the structural proteins of the virus have now been determined. Furthermore, the complete amino acid sequences of these proteins have been deduced. Antigenic sites on the virus proteins (especially the G protein) have been mapped using specific panels of monoclonal antibodies (*113–117*). Antigenic determinants in the G protein and RNP recognized by virus-specific B and T cells have been identified (*118, 119*), and synthetic peptides incorporating these determinants have been tested for antigenicity and immunogenicity in mice (*120*).

In addition to diagnosis and epidemiology, monoclonal antibodies have become essential tools in rabies research for mapping specific epitopes (Chapter 12). More recently, monoclonal antibodies of murine origin have been tested for post-exposure treatment of animals (*121*). Studies are now under way to prepare chimeric (murine–human) antibodies and also to "humanize" monoclonal antibodies of murine origin (*48, 122*).

Expression of the rabies proteins (particularly the G protein) is now widely used in the production of rabies vaccines for the oral immunization of foxes and racoons. Both rabies G-protein and nucleoprotein baculovirus recombinants are now available and could be considered for the production of low-cost rabies vaccines for animals and of rabies reference preparations (*123, 124*) (see also Chapters 34 and 35).

Many laboratories have applied techniques for the cloning and preparation of nucleic probes to the detection of viral nucleic acid target sequences in clinical specimens (*125*). Amplification of the viral RNA by the polymerase chain reaction (PCR) followed by analysis of the amplified sequences was used. Studies of the variability of selected genomic areas between virus strains can be performed on PCR-amplified nucleic acid sequences by enzyme restriction analysis or direct sequencing. The technique has potential for studying the molecular epidemiology of rabies and rabies-related viruses (*50, 126*) (see also Chapter 13). It has recently been used in pathogenicity studies on rabies (*127, 128*), but because of technical difficulties, there are severe limitations on its use as a routine procedure.

Conclusion

Most of the future developments in rabies vaccine production, diagnostic procedures and studies on the pathogenicity of the virus will stem from research carried out using the above techniques. While it is not possible to describe all of these techniques in detail in this book, the following chapters describe those considered essential for the implementation and evaluation of rabies control activities in dogs and wildlife. Adoption of the techniques best suited to local conditions should lead to a marked improvement in the diagnosis of rabies, the control of reference materials and virus strains, and the production of rabies vaccines.

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CHAPTER 3

Characteristics and molecular biology of the rabies virus

N. Tordo[1]

Introduction

Since the early 1980s, the availability of molecular biology techniques has made possible a fundamental examination of the causative agents of many diseases, including rabies. The purpose of this chapter is to review current knowledge of the characteristics of the rabies virus, to summarize the progress made during the past decade and to attempt to delineate the main challenges for the future.

The viruses that cause rabies encephalitis belong to the *Lyssavirus* genus of the Rhabdoviridae family. They were originally classified in four serotypes on the basis of serological and antigenic relationships: serotype 1 comprised the "classical" rabies viruses, notably the "street" (wild) and vaccinal strains; serotypes 2, 3 and 4 were rabies-related viruses prototyped by Lagos bat, Mokola and Duvenhage viruses, respectively. The vaccinal strains of serotype 1 have little or no protective effect against rabies-related viruses. Genetic studies have confirmed and extended this classification (*1*). Four genotypes corresponding to the four serotypes have been characterized. In addition, the recently identified European bat lyssaviruses (EBL1 and EBL2) have been classified in genotypes 5 and 6.

Morphology and structure

Morphology

The virions or virus particles have a bullet-shaped structure (Fig. 3.1), with a diameter of 75 nm and a length of 100–300 nm (*2–6*). Variations in the length can be observed between rabies strains (e.g. CVS is usually longer than PV), or can reflect the presence of defective interfering (DI) particles, which occur when the multiplicity of infection is high. The DI particles possess a truncated genome and are therefore defective in various viral functions, and must depend upon infectious virions to complement their deficiency. Since their smaller genomes replicate rapidly, these particles compete efficiently with normal genomes for encapsidation into the virion.

The virion can be divided into two structural units (Fig. 3.2): a central and dense cylinder formed by the helical ribonucleocapsid, and a thin surrounding envelope (8 nm wide) covered with spike-like projections, which are 10 nm in length and 5 nm apart. The helical ribonucleocapsid is extremely compact, as indicated by the huge random ribbon escaping from the flat end of partially degraded virions.

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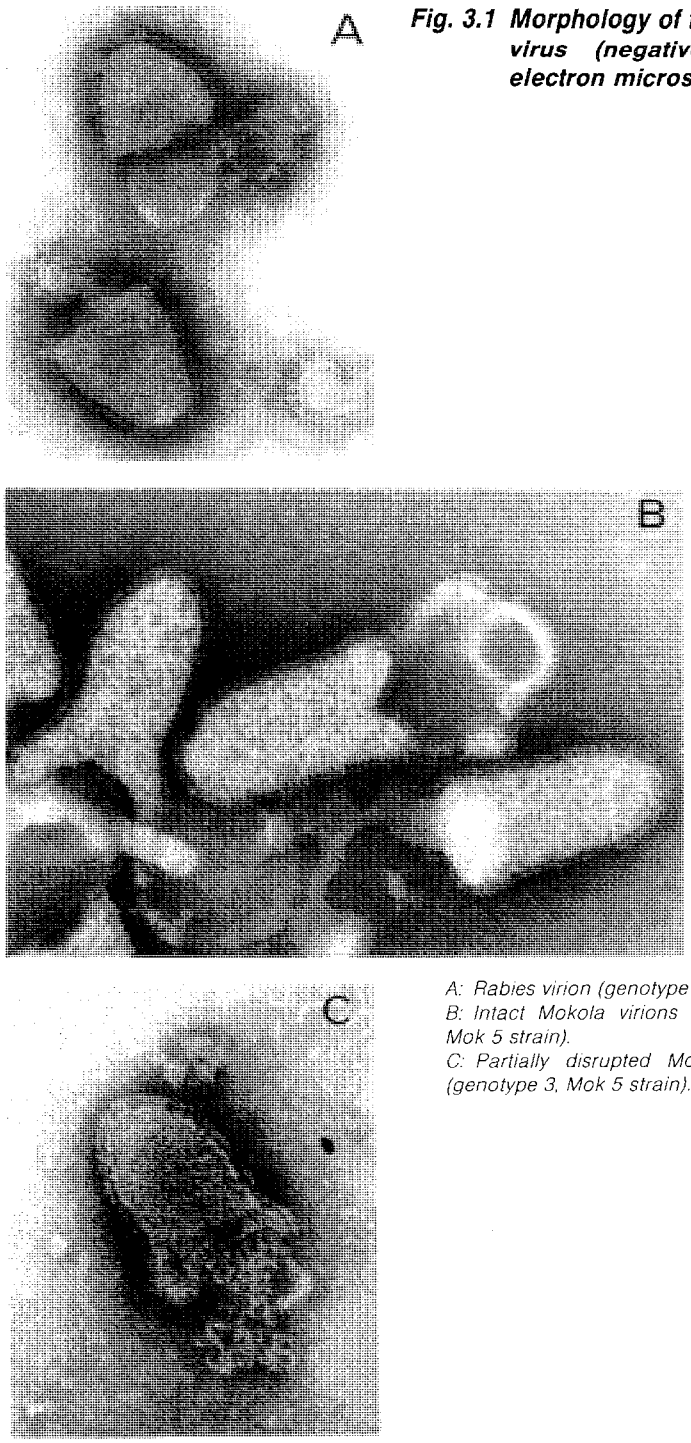
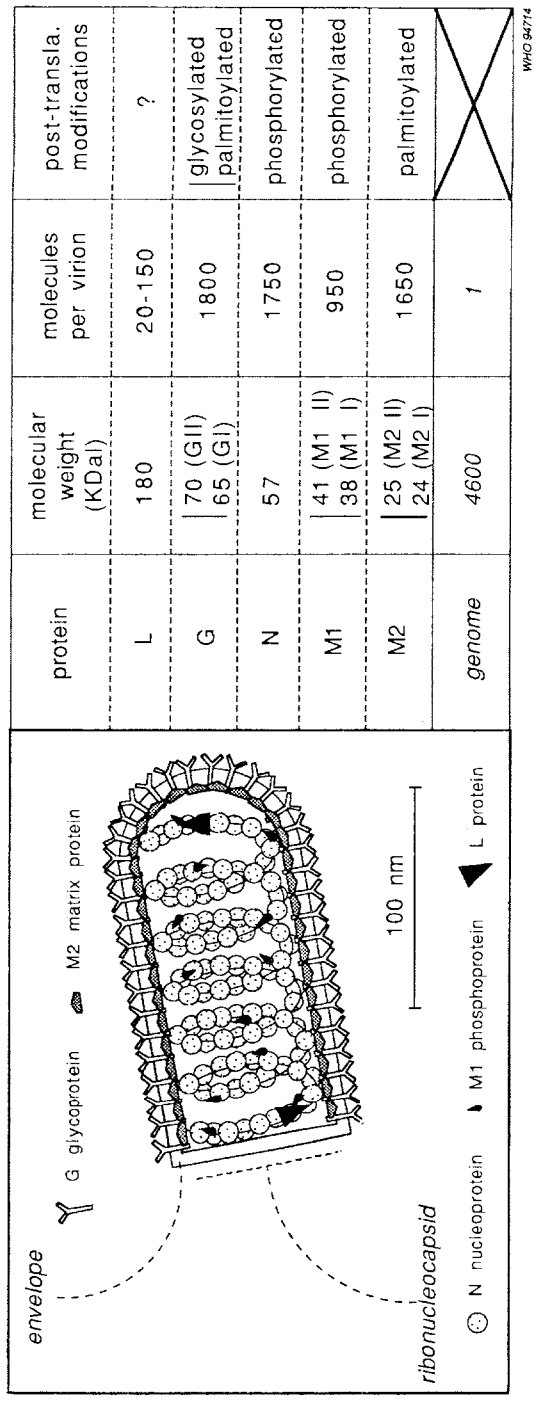


Fig. 3.1 Morphology of the rabies virus (negative-staining electron microscopy)

A: Rabies virion (genotype 1, PV strain).
B: Intact Mokola virions (genotype 3, Mok 5 strain).
C: Partially disrupted Mokola virions (genotype 3, Mok 5 strain).



protein	molecular weight (KDal)	molecules per virion	post-transla. modifications
L	180	20-150	?
G	70 (GII) 65 (GI)	1800	glycosylated palmitoylated
N	57	1750	phosphorylated
M1	41 (M1 II) 38 (M1 I)	950	phosphorylated
M2	25 (M2 II) 24 (M2 I)	1650	palmitoylated
genome	*4600*	*1*	

Fig. 3.2 Structure of the rabies virion

Structure

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

1. The RNA-dependent RNA polymerase (L protein), which has a high relative molecular mass.
2. The glycoprotein (G), which exists in two forms with different extents of glycosylation, both of which are fatty acylated with palmitic acid.
3. The nucleoprotein (N), which is phosphorylated.
4. A phosphoprotein (M1), which has two forms with different extents of phosphorylation.
5. The matrix or membrane protein (M2), which has two conformations differing in the number of internal disulfide bridges. Both forms are palmitoylated.

The relative molecular masses and the number of copies of each protein per virion vary slightly between studies, owing to differences in both the estimation techniques used and in the viral strains studied. The values given in Fig. 3.1 are averaged from numerous reports (*6*). A more recent report proposes slightly different values (*7*).

Proteolytic treatment of the virion with trypsin removes the spike-like projections and selectively affects the G protein (*8, 9*). Only a small G peptide remains linked with the spikeless particle, indicating that the G protein is membrane-anchored and constitutes the main component of the viral spikes. Each spike consists of three associated G proteins (*10*).

Following treatment with hypotonic buffer and edetic acid[1] or non-ionic detergents such as octoxinol or octylglucoside, the viral envelope is solubilized and becomes permeable to trypsin. The associated G protein is released first, followed by fractions of the M2 protein. This finding initially suggested that the M2 protein was embedded in the inner layer of the membrane. However, recent studies on vesicular stomatitis virus (VSV) indicate that only part of the M2 protein is in contact with the membrane, the rest being embedded in the centre of the ribonucleocapsid coil (*11*). Subsequent treatment of the remaining ribonucleocapsid with an ionic detergent such as deoxycholate releases the M1 and L proteins, but does not affect the association between the RNA genome and the N protein. This latter is so intimate that the genome remains insensitive to digestion by ribonucleases.

Despite their parting in distinct units, the five viral proteins are close enough to be experimentally linked by chemical reagents. Whatever its exact position, the M2 protein plays a crucial intermediate and catalytic role within and between the viral envelope and the ribonucleocapsid. The M1 protein was originally considered as a component of the viral membrane before being reassessed as belonging to the ribonucleocapsid. However, the nomenclature "M1" (for membrane) has not been modified.

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[1] Also known as ethylenediamine tetraacetate or EDTA.

Functional analysis of the infection

A productive infection results from a chain of events, consisting first of encounter between the virus and the host, then their interaction and finally multiplication of the virus.

Host species

The maintenance of rabies is ensured by several wild animal species that serve as reservoirs and vectors of the disease in nature. A vector must be highly susceptible to the virus and able to develop interactions favouring the transmission of the disease before death. Several particularly efficient host–virus combinations have emerged in different geographic regions. Currently, the red fox is the main vector of rabies in Europe, while in North America, the racoon and the skunk are also important. In developing countries, dogs remain the major vectors, although wildlife species are also involved. Bat species serve as the main reservoirs of lyssavirus genotypes 2, 4, 5 and 6 and are also involved in the transmission of genotype 1.

Routes of infection

The rabies virus is usually introduced by a bite wound, although infections have been reported from aerosols and licks on broken skin or mucous membrane (*12*) (see also Chapter 1). The virus is neurotropic and rapidly enters the sensory and motor nerve endings of the peripheral nervous system (*13, 14*). Therefore, the rabies virus is only transiently exposed to the immune system although a recent paper suggests that antibody-mediated clearance of rabies from the central nervous system may occur (*15*). Once in the neuron, the virus is transported in the axons by retrograde axoplasmic flow to the perikaryon, where it undergoes replication. The brain stem is infected first, followed by the thalamus and then the cortex (*13*). During the later stages of infection, however, the entire central nervous system is infected as well as certain external tissues, such as the salivary glands, that ensure the transmission of infection. It is not yet clear when the virus starts to replicate. Experimental data support either a primary multiplication in the muscular cells (*16, 17*) or, on the contrary, no multiplication outside the neuronal cells (*14, 18*).

This progressive infection explains why rabies has such a variable incubation period (generally 1–3 months), contrasted by a short symptomatic period (less than 1 week). The latter invariably leads to death because of the absence of effective therapy (*19*). The causes of death remain obscure, as only minor histopathological changes appear to occur. In mice, the virus has been shown to cause electrophysiological dysfunction and alterations in sleep, notably at the level of rapid eye movement sleep (*20*).

Penetration into the host cell

The binding of the rabies virus to the cell membrane is thought to be mediated by the G protein. However, a rabies-specific receptor remains to be characterized. 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 (*21–23*). However, this hypothesis may apply only to muscular cells. For neuronal and fibroblastic cells, it has been shown that oligosaccharides and lipoprotein components such as the sialic acids of gangliosides may also be involved (*13, 24*). The rabies receptor appears to be complex and may vary from one cell type to another.

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.

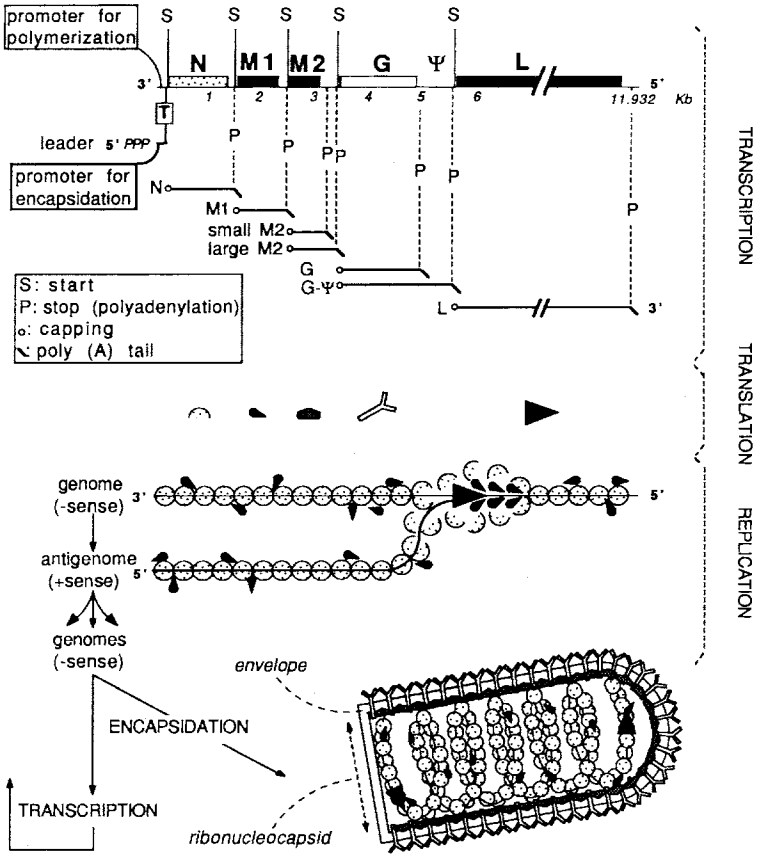
Transcription, replication and budding

The rabies virus possesses an unsegmented negative-stranded RNA genome. All viruses presenting this genome structure belong to three families, the Rhabdoviridae, the Paramyxoviridae and the Filoviridae (*25*). Despite a ubiquitous host distribution, these viruses have evolved an identical expression strategy. 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. Typically, the viral polymerase does not recognize a naked RNA template, but rather one encapsidated with the N protein, as the genome does not undergo uncoating into the cytoplasm.

The mechanisms of transcription, replication and expression of unsegmented negative-stranded RNA genomes (including that of the rabies virus) were originally established from studies on VSV (*6, 26–29*). It is thought that a single promoter for polymerization is recognized by the transcriptase near the 3′-end of the genome (Fig. 3.3). From that point the transcriptional complex progresses towards the 5′-end, producing consecutive monocistronic transcripts: first, one small uncapped, non-polyadenylated leader RNA; then, five mRNAs coding successively for the N, M1, M2, G and L proteins. To control this sequential progression, the transcriptional complex recognizes start (S) and stop or polyadenylation (P) transcription signals flanking the cistrons. These consensus sequences are approximately ten nucleotides in length. The complex is thought to dissociate from the template at each stop signal and to re-initiate poorly at the next start signal. This may be partly due to the size of the non-transcribed intergenic region, which could impair the accessibility of the complex to the start signal. This mechanism results in a progressive decrease in the rate of transcription from the 3′- to the 5′-end of the genome, suggesting that the genomic location of a cistron directly influences its rate of transcription.

A typical feature of transcription of the rabies genome is the phenomenon of alternative termination due to the presence of two consecutive stop signals for the M2 and G cistrons (*30*). Both signals can be alternatively used to produce either a large or a small messenger. Because the transcription complex is thereby released nearby or far upstream from the next start signal, alternative termination influences the efficiency of transcription of the distal gene by modifying the size of the non-transcribed intergenic region. The ratio between the large and the small messen-

Fig. 3.3 Transcription and replication of the rabies genome



ger varies during the course of infection, and is different in fibroblastic and neuronal cells. This suggests that alternative termination is a mechanism for regulating the expression of the rabies genome, which could be influenced by factors of viral or cellular origin, such as proteins or peptides. The latter hypothesis suggests that the neurotropism of rabies should be investigated at the transcriptional level. Interestingly, alternative termination is only observed with certain fixed laboratory strains of rabies virus, such as PV, Pasteur and ERA. It is absent from the wild isolates sequenced up to now, and also from other fixed strains such as CVS, PM and HEP (*31*).

After transcription, the rabies messengers are processed through the cellular translation machinery. Most of them are expressed in the cytosol by free polyribosomes. However, the G protein is produced as a transmembrane molecule in the rough endoplasmic reticulum and is then transported via the Golgi apparatus to the cytoplasmic membrane of the cell, the glycosylated part facing the exterior.

It is only after translation of the mRNAs into rabies proteins that the replication step can begin. This leads to the synthesis of a positive-stranded antigenome that serves, in turn, to amplify negative-stranded genomes for the progeny virions. To

become functional templates for future transcription, the genome and antigenome have to be coated with N proteins. This requires an exact coordination between polymerization and encapsidation. Therefore, a promoter for encapsidation must exist near their 5'-end to initiate the concomitant encapsidation of the growing RNA.

Before viral budding, transcription and replication are inhibited. Simultaneously, there is intense condensation and coiling of the ribonucleocapsids. These events take place in specific areas of the cell membrane where transmembrane G proteins have been concentrated. The virion leaves the cell by budding, the lipidic envelope being pulled out from the host-cell membrane.

The ribonucleocapsid complex, which consists of the RNA genome and the N, M1 and L proteins, is the minimal virion structure exhibiting RNA synthesis. The respective role of each viral polypeptide in this process has been studied less deeply than the mechanisms themselves. On the basis of studies on VSV, it is known that the M1 and L proteins are the catalytic elements involved in the polymerase function. The L protein is the actual RNA-dependent RNA polymerase possessing most of the required enzymatic activities, including RNA synthesis, capping, polyadenylation, and partial kination of the N and M1 proteins. The role of the M1 protein is probably more regulatory: it helps the RNA-dependent RNA polymerase so that it binds correctly to the promoter for polymerization; it possibly uncoats the RNA template upstream of the polymerization complex; and it checks the amount of N protein available for encapsidation.

The N protein plays a major structural role in encapsidating the RNA genome and antigenome. It is therefore involved in the switch between transcription and replication, as suggested by several studies on VSV. These studies indicate that replication cannot begin in the absence of sufficient quantities of N protein to encapsidate the growing template. As long as the amount of N protein remains below a certain level, the transcription complex recognizes a stop signal (T) at the end of the leader gene and releases the leader RNA. It then recognizes the start and stop signals flanking the N, M1, M2, G and L genes and produces the naked mRNAs. When there are sufficient amounts of N protein, the transcription complex participates in a concurrent assembly of the growing template and somehow is responsible for the polymerase ignoring the stop signal. This event corresponds to the switch between transcription and replication. The viral polymerase then becomes unable to recognize the other start and stop transcription signals. Since the leader RNA is no longer produced, the promoter for encapsidation remains linked to the 5'-end of the growing genome, which is progressively encapsidated in a cooperative manner.

The M2 protein plays an important role during the latter stages of infection, notably during morphogenesis. Its position, between the ribonucleocapsid and the membrane, permits its interaction with both internal (N protein) and external (cytoplasmic tail of the G protein) proteins. It is able to inhibit RNA synthesis, mediate the coiling of the ribonucleocapsid, and concentrate the G protein, all of which are required before the virion buds out of the infected cell.

Viral proteins involved in the host immune response

Although all the viral proteins show antigenicity, they do not all play the same role in protection (*32*). The purified G protein has been shown to protect against an

intracerebral challenge with rabies virus, while the purified ribonucleocapsid only protects against a peripheral challenge (*33*). The G protein is the only rabies antigen that consistently induces virus-neutralizing antibodies (*34*). This property mainly depends on the preservation of its three-dimensional structure, although a linear neutralizing epitope has been identified (*35*). On the other hand, it shares the capacity to induce a cellular immune response involving both T-helper cells and cytotoxic T cells with the N and M1 proteins. The T-cell response is thought to play an important role in the immune response to rabies (*36*).

These studies suggest that the G protein is the most important antigen for immunization. However, the N protein is also important for two principal reasons: (i) because of its capacity to substantially enhance the T-helper cell immune response to rabies vaccination; and (ii) because it is less variable than other antigens. This suggests that the N protein is the best candidate to increase the protection spectrum of vaccines, notably to distant rabies-related viruses (*1, 33*).

Molecular biology of the rabies virus

Since 1981, when the glycoprotein mRNA of the ERA strain became the first rabies gene to be cloned and sequenced (*37*), considerable advances have been made in determining the sequence of the rabies genome (Fig. 3.4). Two basically distinct strategies were adopted: (i) cloning the viral mRNAs; (ii) cloning the RNA genome directly. The latter strategy also enabled the non-transcribed intergenic regions to be studied. As shown in Fig. 3.4, the only rabies (genotype 1) genomes that have been completely cloned and sequenced are those of the PV strain (11 932 bases) (*38–40*) and the SAD-B19 strain (11 928 bases) (*41*). To date the rabies-related strains have been only partially sequenced (Fig. 3.5).

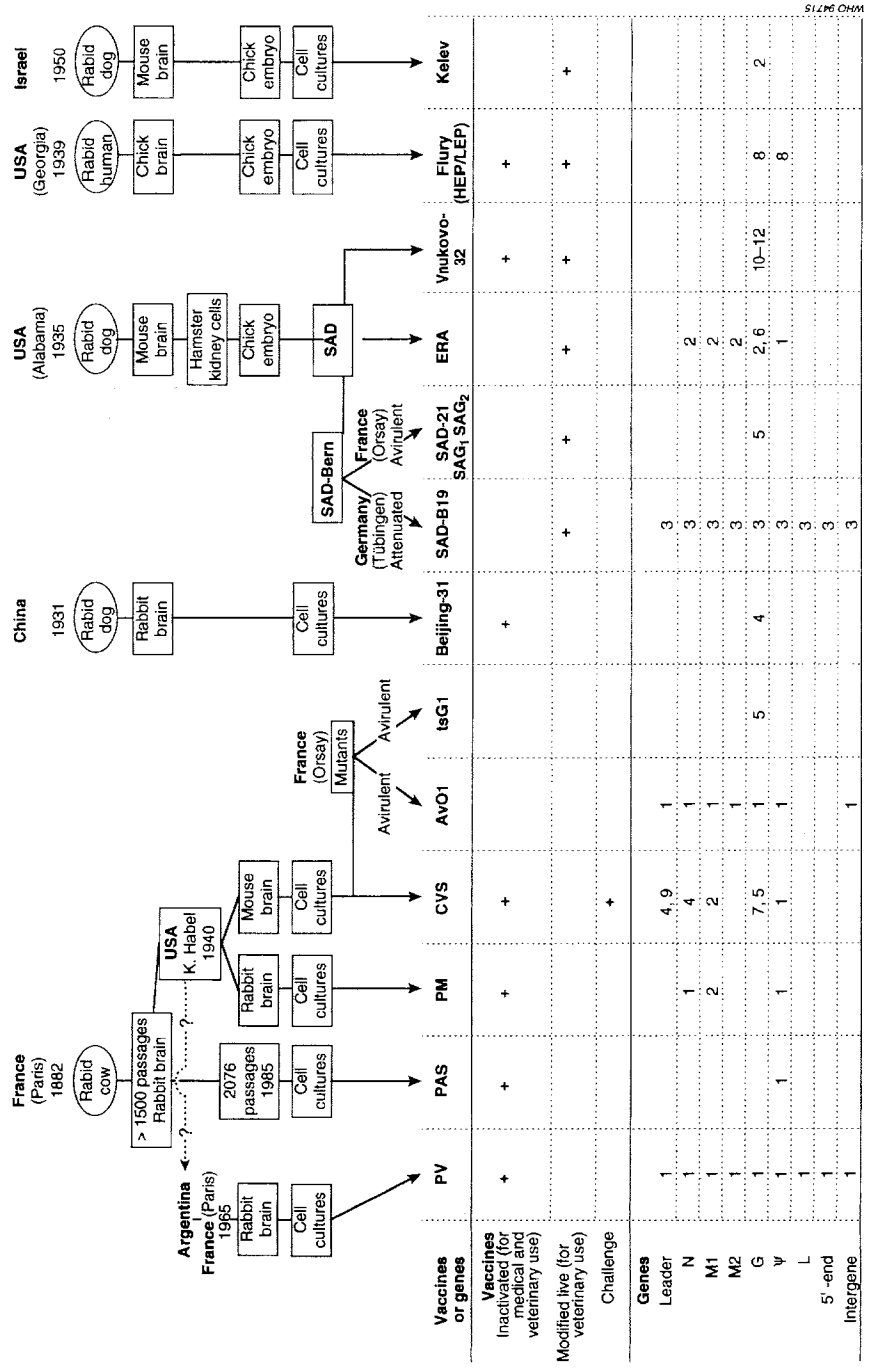
With the development of the polymerase chain reaction (PCR), it has now become possible to amplify any rabies gene (*42, 43*). The sensitivity of this technique permits the isolation of the original rabies strains present in the infected tissue, without any cell culture or brain adaptation. A new technique combining PCR amplification and direct sequence analysis has been developed (*42*) (see also Chapter 13). This powerful tool of molecular epidemiology is currently leading to a dramatic increase in our fundamental knowledge of the lyssaviruses.

Considerable progress has also been made in expressing the rabies genes, since they are all now available. Fundamental aspects have been investigated through the expression of the L protein, in order to evaluate its role during transcription and replication (*44*). With this exception, most of the studies carried out have focused on the development of a recombinant vaccine expressing the genes coding for the G and N proteins, which are involved in the immune response to rabies infection (*45*) (see also Chapter 35).

The analysis of sequence data has led to substantial progress in understanding the structure–function relationships of the various elements of the rabies virus (*6, 24*). It has also led to an analysis of the evolution of the *Lyssavirus* genus (*1*), and more generally of the Mononegavirales order (*46*).

The leader RNA

This is a small RNA (57–58 ribonucleotides), which is very rich in A residues transcribed at the 3'-end of the genome. During transcription it carries the

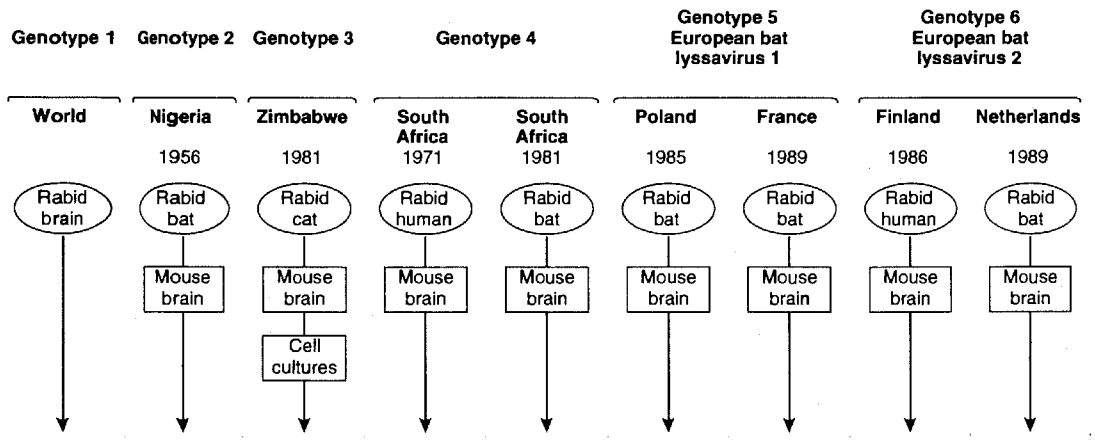


Vaccines or genes	PV	PAS	PM	CVS	AvO1	tsG1	Beijing-31	SAD-B19	SAD-21 SAG_1 SAG_2	ERA	Vnukovo-32	Flury (HEP/LEP)	Kelev
Vaccines													
Inactivated (for medical and veterinary use)	+	+	+	+			+				+	+	
Modified live (for veterinary use)								+	+	+	+	+	+
Challenge				+									
Genes													
Leader	1			4, 9	1			3					
N	1		1	4	1			3		2			
M1	1		2	2	1			3		2			
M2	1				1			3		2			
G	1			7, 5	1	5	4	3	5	2, 6	10–12	8	2
ψ	1	1	1	1	1			3		1		8	
L	1							3					
5' -end	1							3					
Intergene	1				1			3					

1. WHO Collaborating Centre for Reference and Research on Rabies, Pasteur Institute, Paris, France.
2. WHO Collaborating Centre for Reference and Research on Rabies, The Wistar Institute of Anatomy and Biology, Philadelphia, PA, USA.
3. WHO Collaborating Centre for Rabies Surveillance and Research, Federal Research Institute for Animal Virus Diseases, Tübingen, Germany.
4. WHO Collaborating Centre for Reference and Research on Rabies, Center for Infectious Diseases,Centers for Disease Control, Atlanta, GA, USA.
5. Centre National de la Recherche Scientifique, Gif-sur-Yvette, France.
6. Connaught Centre for Biotechnology Research, Willowdale, Ontario, Canada.
7. Genentech, San Francisco, CA, USA.
8. Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan.
9. Medical College, Oita, Japan.
10. Agricultural Biotechnology Center, Gödöllö, Hungary.
11. Russian Academy of Sciences, Pushkino, Russian Federation.
12. Academy of Medical Sciences, Moscow, Russian Federation.

[a] The strains listed are those that have been partially or totally sequenced.
[b] Numbers refer to laboratories where the sequences were performed.

Fig. 3.5 Wild isolates of rabies (genotype 1) and rabies-related viruses (prototypes of genotypes 2–6) that have been studied at molecular level [a,b]



Genes	Rabies	Lagos bat	Mokola 5 (Mok5)	Duvenhage 1 (Duv1)	Duvenhage 2 (Duv2)	EBL 1	EBL 1	EBL 2	EBL 2
Leader			1						
N	1, 4	1	1	1	1	1	1	1	1
M1			1						
M2			1						
G	1, 5	1	1	1	1	1	1	1	1
ψ	1		1						
L			1						
5' -end			1						
Intergene			1						

1. WHO Collaborating Centre for Reference and Research on Rabies, Pasteur Institute, Paris, France.
2. WHO Collaborating Centre for Reference and Research on Rabies, The Wistar Institute of Anatomy and Biology, Philadelphia, PA, USA.
3. WHO Collaborating Centre for Rabies Surveillance and Research, Federal Research Institute for Animal Virus Diseases, Tübingen, Germany.
4. WHO Collaborating Centre for Reference and Research on Rabies, Center for Infectious Diseases, Centers for Disease Control, Atlanta, GA, USA.
5. Centre National de la Recherche Scientifique, Gif-sur-Yvette, France.
6. Connaught Centre for Biotechnology Research, Willowdale, Ontario, Canada.
7. Genentech, San Francisco, CA, USA.
8. Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan.
9. Medical College, Oita, Japan.

[a] The strains listed are those that have been partially or totally sequenced.
[b] Numbers refer to laboratories where the sequences were performed.

promoter of encapsidation and cleaves it from the distal mRNA transcripts. In VSV, the leader RNA has also been implicated in the shut-off of the host-cell macromolecular synthesis (*47*). However, this effect is not usually observed during infection with the rabies virus (*48*).

The N protein

This is a 450-amino acid long polypeptide which is phosphorylated on a serine residue in position 389 (*49*). It shows a segmented homology with the N protein of VSV, involving mainly the central part of the protein (*38*). The stretches of conserved amino acids are probably those directly interacting with the RNA genome, since one function of the protein is to encapsidate and protect the genome.

Monoclonal antibodies directed against the N protein have been used to differentiate and classify rabies wild isolates into types of lyssaviruses (*50–52*) (see also Chapter 12). Three antigenic sites have been characterized along the protein, but only two of them are mapped. Sites I and III involve the stretches of amino acids in position 374–383 and 313–337, respectively. Several immunodominant T-helper epitopes have also been characterized (*53*), one of which (in position 404–418) is protective for mice when coupled with the linear epitope of the G protein (*54*).

The M1 protein

This highly hydrophilic protein is 297 and 303 amino acids long in rabies and Mokola virus, respectively. It presents different phosphorylation states (*55*) and possesses numerous serine and threonine amino acids, which anchor the phosphate residues. Phosphorylation provides an overall negative charge which is increased by the very high content of acidic (aspartate and glutamate) amino acids. The primary nucleotide sequence of the M1 protein is very poorly conserved between rabies virus and Mokola virus, particularly in the central part (position 55–200).

Two antigenic sites have been characterized along the M1 protein, both located in position 75–90. Recently, immunodominant cytotoxic T epitopes and T-helper epitopes were identified in position 191–206 (*56*). Interestingly, they are located partly in the poorly conserved central region (position 191–197), and partly in the conserved carboxy side (position 198–206).

The M2 protein

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. As with VSV, the 40 amino terminal residues, rich in charged and proline residues (*57*), could mediate the inhibitory effect on transcription and replication before the ribonucleocapsid is coiled. This region seems to be involved in the host immune response to rabies, since a major antigenic determinant was recently located between residues 1 and 72 (*58*). A 19-residue central segment (in position 89–107) appears sufficiently hydrophobic to anchor

the protein into the virion membrane (*39*). The palmitoylation site of the protein, which probably involves a cysteine residue, remains to be characterized (*59*). However, it does not appear to be in direct proximity to the presumed membrane-binding site. As discussed earlier, recent studies on VSV suggest that the M2 protein extends from the inner layer of the viral membrane to the internal core of the ribonucleocapsid coil (*11*). Regardless of its exact location, the M2 protein is believed to play an important role in the morphogenesis of the virus.

The G protein

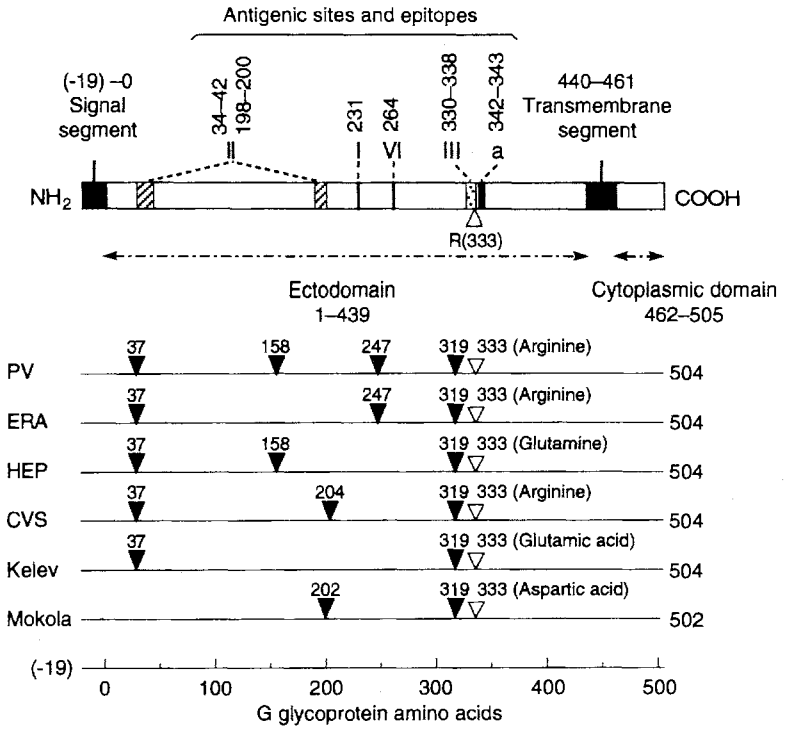
The G protein (Fig. 3.6) is the best studied of the rabies proteins, at both the structural and immunological levels (*6, 24, 32, 50, 60*). It is 524 and 522 amino acids long in rabies and Mokola virus, respectively, and contains two hydrophobic segments typical to its transmembrane nature. The amino terminal signal segment (first 19 residues) initiates the translocation of the nascent protein through the rough endoplasmic reticulum membrane, before being cleaved in the mature protein. The translocation process continues up to the transmembrane segment (position 440–461), which remains anchored in the membrane as indicated by the palmitoylation of the cysteine residue in position 461 (*59*). This segment separates the cytoplasmic carboxy terminal domain (position 462–505), which interacts with the internal proteins, from the external glycosylated amino terminal domain (position 1–439). The glycosylation and the fatty acylation of the protein take place during its transport from the rough endoplasmic reticulum to the Golgi apparatus and to the cytoplasmic membrane. One glycosylation site (in position 319) appears to be of major importance, both because it is present (and probably used) in all the lyssavirus strains sequenced up to now, and because it is the only region that shows homology with the G protein of VSV (*61*). The other sites vary between different strains and are not systematically used.

At least eight antigenic sites have been located on the external domain of the G protein of different rabies strains (I–VI, "a" and G1) (*35, 60, 62–65*). With the exception of sites VI and G1, which were also identified under denaturating conditions, these sites depend on conformation and the folding of the protein. Only five sites have been definitively mapped. Sites I, III, VI and "a" involve the amino acids in position 231, 330–338, 264 and 342–343, respectively. Site II is discontinuous and involves two separate stretches in position 34–42 and 198–200 linked by a disulfide bridge. It has been suggested that the current nomenclature should be modified according to the relative importance of the G protein regions in stimulating the B-cell response (*60*). The term "antigenic site" would be reserved for regions described by numerous different monoclonal antibodies (sites II, III and "a"), while the word "epitope" would be used for sites recognized by only one monoclonal antibody (epitopes I, IV, V, VI and G1). It is noteworthy that the sequences corresponding to the antigenic sites of the rabies G protein appear to diverge in the G protein of Mokola virus.

T epitopes have also been located along the G protein using chemically cleaved or synthetic peptides (*66, 67*).

The G protein is also involved in the pathogenesis of rabies and is believed to assume at least part of the neurotropism of the virus. In genotype 1 viruses, neurovirulence seems to be directly related to the maintenance of an arginine (or lysine) residue in position 333 (site III) (*68*). Mutants with other amino acids in this

Fig. 3.6 Structure, antigenic sites and epitopes of rabies glycoprotein[a]



▼ Potential glycosylation sites.

▽ Residue involved in neurovirulence.

[a] The numbering is made on the assumption that the signal segment is cleaved in the mature protein.

position cannot infect certain types of neurons, presumably because they are unable to recognize the receptor (*14, 69*). However, several attenuated vaccinal strains, such as HEP or Kelev, are muted in position 333 (*70, 71*). Furthermore, Mokola virus (genotype 3), which is highly neuropathogenic in mice and causes more severe encephalitis than rabies virus in dogs and monkeys, possesses an aspartate residue in position 333 (*72*). This suggests that tissue specificity is probably very complex.

The L protein

This 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. Because only minute amounts are present in the virion and in the infected cell, it is the least studied of the rabies proteins at the biochemical and immunological level, but the best analysed at the theoretical level.

One of the main characteristics of the L protein is the sequence homology that it shows with the L protein of other members of the Mononegavirales order, which suggests that they have evolved from a common ancestor (*40, 73*). The homology is not randomly distributed along the protein, however, and some domains are highly conserved, with the amino acids in identical positions, while others are more variable. Such a distribution of independent conserved domains joined by more variable areas is consistent with the multifunctional nature of the L protein (*40, 73*). It suggests that different activities are linked within the final polypeptide, where they may retain a certain degree of independence, as indicated by the complementation observed between VSV L-protein mutants. Several activities were tentatively attributed to certain domains, providing the first available guidelines for the future dissection of the functions of the L protein by site-directed mutagenesis (*73*).

It is noteworthy that the key domain catalysing the polymerization of the rabies genome ("polymerase module") has been mapped. The "polymerase module" appears ubiquitous in the animal kingdom, since it is similar in all the RNA-dependent polymerases (RNA polymerases and reverse transcriptases) and partly conserved in the DNA-dependent polymerases (*74, 75*). These results illustrate how rabies research can have influence beyond its own domain.

Genomic signals

The start and stop transcription signals (internal signals) flanking the cistrons of the rabies and Mokola genome have been determined by S1 nuclease mapping experiments (*6, 39*). They are composed of 9 nucleotide long consensus sequences closely related to those of VSV. The stop signal is terminated by a sequence of 7 uridine residues, which are reiteratively copied by the transcriptase in order to produce the polyadenylation tail of each mRNA before re-initiating at the next start signal.

The promoters for polymerization and for encapsidation (external signals) are assumed to be present in the first 11 nucleotides of the 3'- and 5'-ends of the genome, respectively (*40, 72, 76*). These sequences are strictly conserved and are inversely complementary. However, the genome is not likely to generate a panhandle structure during transcription or replication, since the genomes and antigenomes are encapsidated as soon as they are synthesized.

Within a viral genus (*Lyssavirus* or *Vesiculovirus*), all the signals are strongly conserved. However, when the whole Rhabdoviridae family is considered, the internal signals appear more stable than the external ones (Fig. 3.7).

The intergenic regions

The intergenic regions of the rabies and Mokola virus genomes (Fig. 3.7) vary both in length (2–24 nucleotides) and nucleotide composition (*1, 39, 72*), while those of the VSV genome (Indiana strain) are conserved (mostly GA). This variation could modify the progressive loss of transcriptional efficiency occurring between cistrons, and partly explain the weaker performance of rabies virus compared with VSV during cell infection: slower cycle of transcription and replication, less production, and virtually no inhibition of cellular synthesis.

Fig. 3.7 Genomic signals of rhabdoviruses

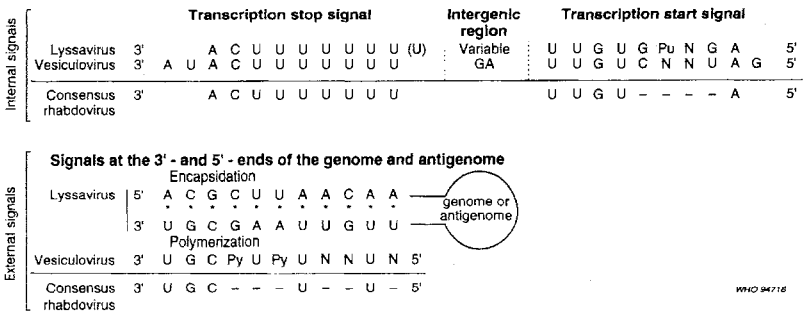
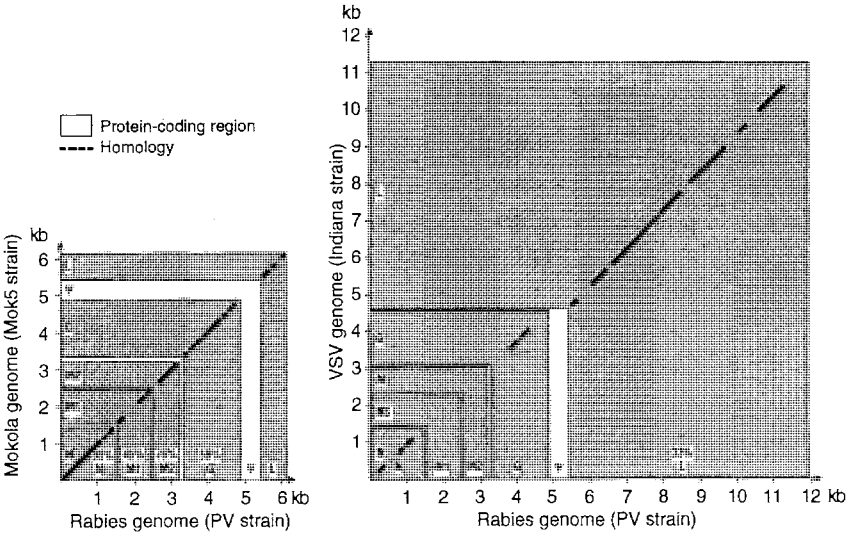


Fig. 3.8 Comparison of the genome of rabies virus (PV strain) with those of Mokola virus (Mok 5) and vesicular stomatitis virus (Indiana strain)



Evolution of the rabies virus

A comparison of the genome of the rabies virus (PV strain) with those of the Mokola virus (Mok 5) and VSV (Indiana strain) clearly indicates that unequal selective pressures were imposed on the viral sequences during evolution (Fig. 3.8). Between rabies and Mokola genomes, representing the two more divergent genotypes of the *Lyssavirus* genus, the homology decreases in the following order: N protein (80% of conserved amino acids), M2 protein (76%), G protein (58%) and M1 protein (45%). As the Mokola polymerase has not yet been totally sequenced , it is not currently possible to evaluate its divergence rate. However, from a larger evolutionary distance, such as that existing between rabies and VSV genomes, representing prototypes of the two genera of the Rhabdoviridae family, the

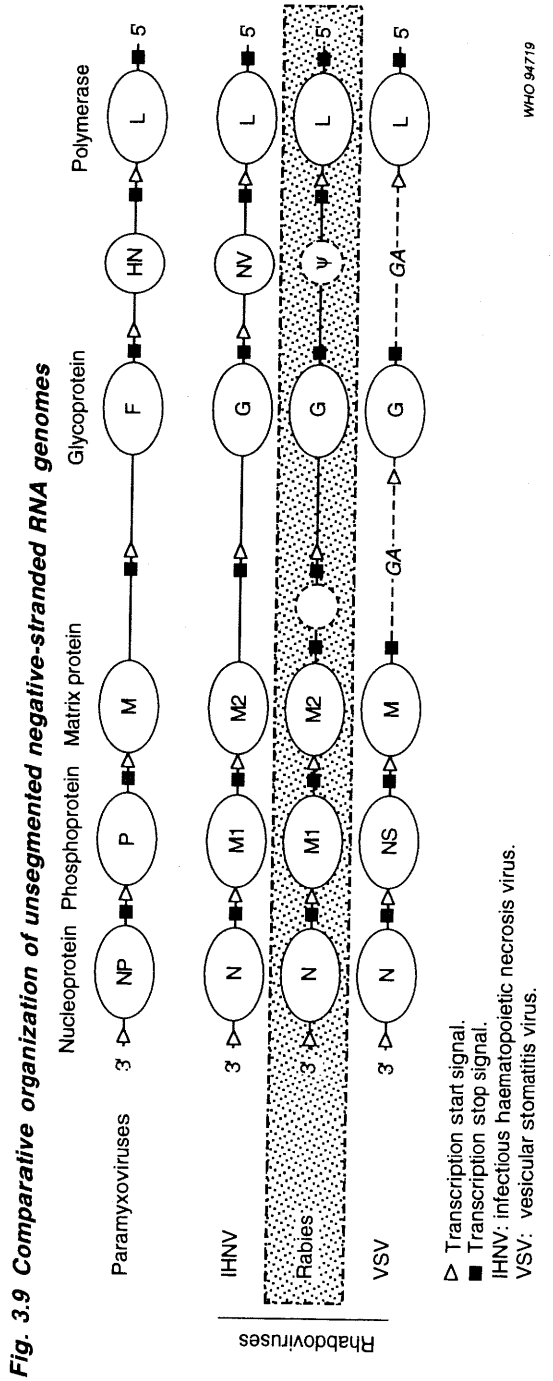


Fig. 3.9 Comparative organization of unsegmented negative-stranded RNA genomes

polymerase remains the only conserved protein (33% of conserved amino acids), together with stretches of the N and G proteins, as discussed previously.

It seems, indeed, that the need for conservation of a precise scenario for genome expression has led to a stringent maintenance of the major controlling elements, such as:

— the polymerase encoding the enzymatic activities required for transcription and replication;
— the genomic signals (external and internal);
— the organization of the genome, since the decreasing rate of transcription results in a direct correlation between gene location and expression rate.

Fig. 3.9 shows that the organization of the unsegmented negative-stranded RNA genomes is similar, with the major structural genes (N, M1 and M2 proteins) encoded at the 3′ side, while the 5′ half codes for the L protein (*46*). These locations are adapted to the stoichiometric and catalytic requirements of the respective gene products.

Unsegmented negative-stranded RNA genomes generally use most of their length for coding purposes. In this context the *Lyssavirus* genome is atypical, since two substantial non-coding regions follow the M2 and G genes, respectively. The proximal region separates the 3′ block of structural genes and the glycoprotein gene (G for Rhabdoviridae, F for Paramyxoviridae), while the distal one separates the glycoprotein and the polymerase genes. A comparison of unsegmented negative-stranded RNA genomes indicates that both these regions are located in very plastic areas during evolution (*46*) (Fig. 3.9). This suggests that rabies virus is an evolutionary link and that the regions may represent remnant protein genes. This assumption was mainly developed on the basis of the analysis of the rabies G–L intergene (*6, 39*), which encodes:

1. The HN (haemagglutinin–neuraminidase) protein in paramyxoviruses.
2. The NV protein (unknown function) in the infectious haematopoietic necrosis virus (IHNV), a salmonoid rhabdovirus.
3. The remnant ψ pseudogene in lyssaviruses, which is similar in size to the NV gene.
4. The dinucleotide GA in VSV, which appears at the time of size restriction.

From a mechanistic point of view, it is noteworthy that the two M2–G and G–L intergenes of the rabies virus (PV strain) genome are precisely those where the phenomenon of alternative termination is observed during transcription.

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PART II

Routine laboratory procedures

CHAPTER 4

Rapid microscopic examination for Negri bodies and preparation of specimens for biological tests[1]

E. S. Tierkel[2] & P. Atanasiu[3]

Rabies virus causes the appearance of specific inclusion bodies, known as Negri bodies, in the cytoplasm of infected nerve cells or cell cultures. These inclusion bodies can be detected histopathologically by microscopy or by the fluorescent antibody (FA) test (see Chapter 7). This chapter describes how to prepare samples for these tests.

The techniques employed in the laboratory diagnosis of rabies should embrace optimum conditions of accuracy, speed and economy. The method employing microscopic examination for Negri bodies, using the simple application of brain tissue to a slide and Sellers' technique for staining (see Annex), fulfils these requirements.

It has been found that Negri bodies, when present, are most readily demonstrated in Ammon's horn (hippocampus major) of the brain and also in the pyramidal cells of the cerebral cortex and Purkinje's cells of the cerebellum; they are found to a much more limited extent in the neurons of the thalamus, pons, medulla, spinal cord and sensory ganglia.

Dissection of the brain

A very simple operation is required to expose Ammon's horn, which is generally the best area for demonstration of Negri bodies in most rabid animals. With a pair of sterile scissors, a longitudinal incision is made into the dorsal surface of each cerebral hemisphere, about 2 cm lateral to the longitudinal fissure or midline of the brain (see Fig. 4.1). The incision is made from the region of the occipital pole of the hemisphere and is extended forward for 3–5 cm and downward through the grey matter, and then completely through the white matter until a narrow space, the lateral ventricle, is reached. The opening is then widened by spreading the incised hemisphere, and Ammon's horn will be revealed as a semi-cylindrical, white, glistening body bulging laterally from the ventricle floor (see Figs. 4.2 and 4.3). It has a spiral contour and, on cross-section, a characteristically rolled surface.

Preparation of slides

Slides should be made first from Ammon's horn, then from the cerebral cortex, and finally from the cerebellum. Tissue samples (at least six) should be taken from

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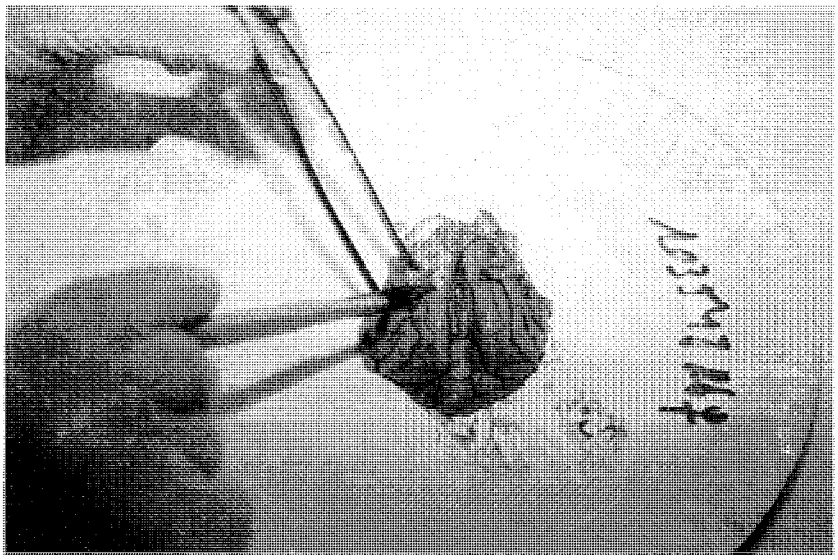
[1] Based on the chapter in the previous edition, which was prepared by the late E. S. Tierkel and updated by the late P. Atanasiu.

[2] Deceased. Former Assistant Surgeon-General, US Public Health Service, Department of Health, Education and Welfare, Washington, DC, USA.

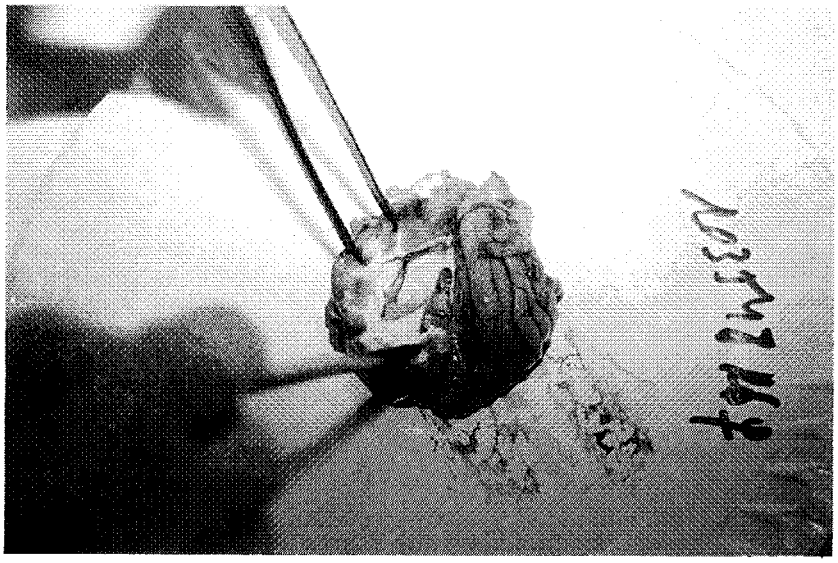
[3] Deceased. Former Honorary Professor, Pasteur Institute, Paris, France.

Fig. 4.1 Site of incision for locating Ammon's horn



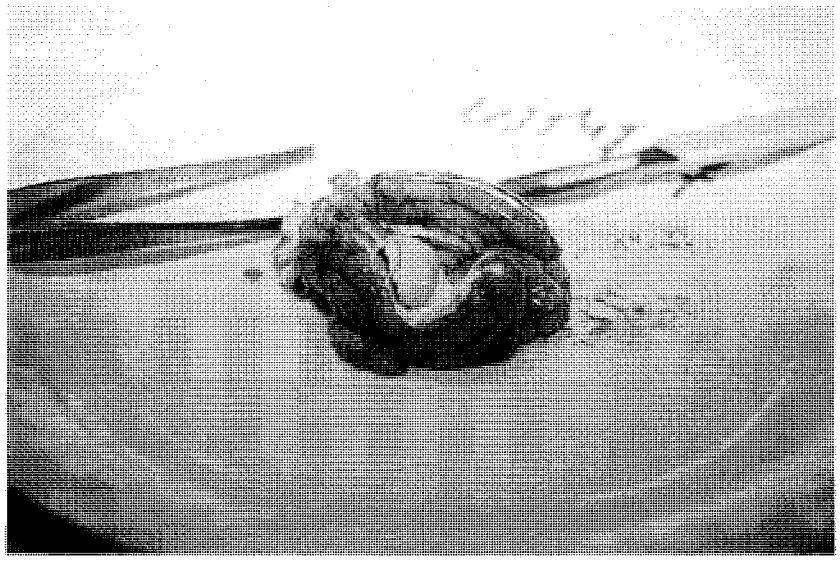
By courtesy of J. Barrat, Laboratoire d'Etudes sur la Rage et la Pathologie des Animaux sauvages, Centre National d'Etudes vétérinaires et alimentaires (CNEVA), Malzéville, France.

Fig. 4.2 Incision to the lateral ventricle, showing Ammon's horn



By courtesy of J. Barrat, Laboratoire d'Etudes sur la Rage et la Pathologie des Animaux sauvages, CNEVA, Malzéville, France.

Fig. 4.3 Close-up of Ammon's horn bulging from the floor of the lateral ventricle



By courtesy of J. Barrat, Laboratoire d'Etudes sur la Rage et la Pathologie des Animaux sauvages, CNEVA, Malzéville, France.

these three areas on each side of the brain and examined microscopically before the brain is reported to be negative for rabies. It is always wise to select another area from each hippocampus for good measure.

The following three methods of applying fresh brain tissue to slides are recommended.

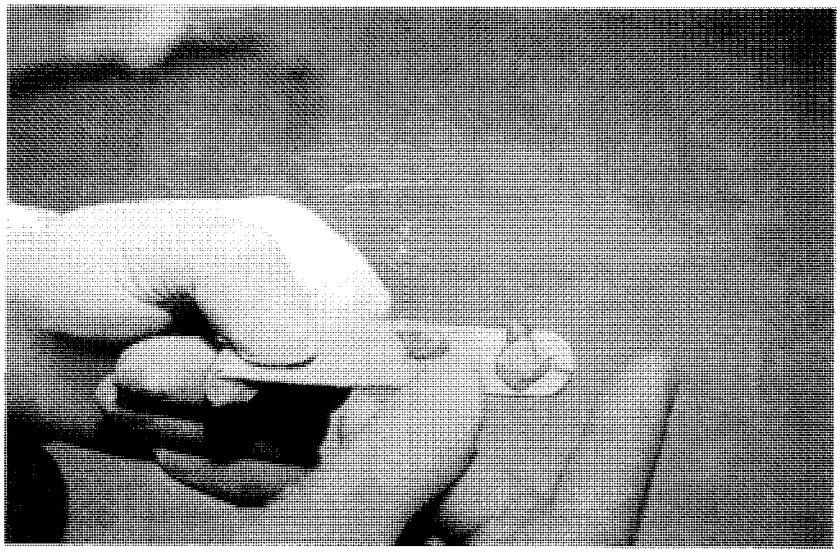
Impression method

With a pair of scissors, small transverse sections (2–3 mm in thickness) of brain tissue (Ammon's horn, cerebrum or cerebellum) are cut and placed on clean blotting-paper or a wooden tongue-depressor, cut surface facing upward (Fig. 4.4). A clean microscope slide is then touched against the cut surface of the section and pressed gently downwards with just enough pressure exerted to create a slight spread of the exposed surface of the tissue against the slide. According to the size of the section, 3–4 impressions can be made on one slide (Fig. 4.5). *While still moist*, the slide is flooded with Sellers' stain for a few seconds, rinsed under the tap and dried at room temperature without blotting. The preparation is then ready for examination. The impression may be examined directly under oil, or covered with a coverslip mounted in balsam. This method is preferred to the others described here because it maximizes the amount of nerve tissue that can be examined and minimizes the amount of cellular damage.

Fig. 4.4 Transferring a specimen (from Ammon's horn and the rachidian bulb) to a wooden spatula (tongue-depressor) before making impressions

By courtesy of J. Barrat, Laboratoire d'Etudes sur la Rage et la Pathologie des Animaux sauvages, CNEVA, Malzéville, France.

Fig. 4.5 Impression method of slide preparation



By courtesy of J. Barrat, Laboratoire d'Etudes sur la Rage et la Pathologie des Animaux sauvages, CNEVA, Malzéville, France.

Smear method

The smear method consists of placing a very small section of brain tissue on one end of the slide. Another slide is used to crush the section of tissue against the first slide and is then drawn across the length of the slide. The result is a fairly homogeneous thin film of tissue covering about three-quarters of the area of the slide. In this technique the tissue is spread over a rather extensive area. Care should be taken not to use too large a tissue-section, as this will result in an excessively thick film, making proper staining and microscopic examination impossible. The impression method, however, gives superior results.

"Rolling" method

The last method, the "rolling" technique, consists of cutting a piece of brain tissue about 5 mm in diameter, and rolling or teasing it gently (cut surfaces downward) over the entire surface of the slide with a toothpick or wooden applicator.

The staining procedure of Sellers is recommended here because of its accuracy and simplicity. In this technique, no preliminary fixation is required, since the tissue film is fixed and stained simultaneously, making it one of the most rapid and easily handled methods.

The Negri body: differential diagnosis

Although generally rounded in form, the Negri body may be found to assume any shape. At various times in different laboratories it has been demonstrated to be round, oval, spheroid, amoeboid, elongate, triangular, etc. By the same token, there is great variation in size; generally it is found to be between 0.24 and 27.0 μm. It is characteristically acidophilic in staining reaction, and takes on the pink to purplish pink/magenta colour in differential stains that use basic fuchsin or eosin with methylene blue as their base (see Plate 5.1,C, page 70).

The Negri body is found within the cytoplasm of the neuron, typically between the nucleus and one corner of the neuron, or in the prolongation of the cell body. However, it should be stressed that the intracytoplasmic position of the Negri body can be expected with reasonable consistency only in histological sections of the brain. In the simple tissue-application techniques described above, the histological pattern is disturbed and one may very often see well-formed Negri bodies that appear to be entirely outside the neuron. Thus, in methods such as the impression, smear or rolling techniques, the intracellular position of the Negri body is not required as a diagnostic criterion, and Negri bodies that satisfy the requisites of morphological identification, whether inside or outside the neuron, are sufficient to establish a positive diagnosis.

The most characteristic feature of the Negri body is its internal structure. It is this feature that serves as the essential criterion for positive identification in the techniques described in this chapter. The matrix of the Negri body has an acidophilic staining reaction, and contained within this magenta-red structure are small inner bodies (*Innerkörperchen*), basophilic granules that stain dark-blue to black. The size of these inner granules generally varies from 0.2 μm to 0.5 μm. Classically, the well-formed Negri body—the so-called textbook picture—will have its inner granules arranged in rosette fashion, with one large centrally placed body

and a series of smaller granules arranged neatly around the periphery of the Negri body. It should be pointed out, however, that this picture is the exception rather than the rule, and it is very rare that such an orderly arrangement of the inner granules is seen. For diagnostic purposes, it is sufficient to establish the presence of these dark-blue/black staining granules, regardless of their numbers or pattern of distribution within the matrix of the Negri body (see Plate 5.1,C, page 70).

There is universal agreement that the Negri body is specific for rabies and its presence always indicates this infection. Furthermore, a fully formed Negri body cannot be confused with other inclusion-like bodies. However, other types of inclusion body are sometimes found in animal brains submitted for diagnosis and, because of certain similarities, may be mistaken for Negri bodies. For example, the acidophilic inclusion bodies of canine distemper or Rubarth's disease (canine infectious hepatitis, fox encephalitis) are occasionally encountered in the brains of dogs and foxes. These seem to occur more often in the thalamus and lentiform nuclei than in the hippocampus. By the same token, the brains of non-rabid cats and laboratory white mice occasionally contain nonspecific acidophilic inclusion bodies when submitted for rabies diagnosis. All these non-rabies inclusion bodies have the same staining characteristics with Sellers' stain (see Annex), and they cannot be differentiated from each other with the techniques described above. However, these non-rabies inclusion bodies, as a group, can be differentiated from Negri bodies with the use of Sellers' stain. The following characteristics may be used as a guide in this differentiation.

Negri bodies	Non-rabies inclusion bodies
Presence of basophilic inner granules	Absence of internal structure[a]
Heterogeneous matrix	Homogeneous matrix
Less refractive	More refractive
Magenta (heliotrope) tinge	Colour more acidophilic (pinker)

[a] See Plate 5.1,D, page 70.

Small atypical intracytoplasmic inclusion bodies are sometimes found in animals killed during the early stages of rabies. For that reason, it is imperative to hold dogs suspected as rabid in quarantine, rather than to kill them immediately and send the brain to a laboratory for diagnosis (see Appendix 1). There are two reasons for this. First, it will permit observation for symptoms of rabies, which may make possible a clinical diagnosis of the disease. Secondly, the longer the animal is allowed to live, the better the chance of obtaining a positive microscopic diagnosis. The length of clinical illness in rabies is directly related to the size, abundance and development of Negri bodies. Thus, if the disease runs its full course, Negri bodies are likely to be more abundant and fully formed with good internal structure.

The mouse inoculation test

Since Negri bodies cannot always be found in the brains of animals dying of rabies, it is important that negative specimens be tested by animal inoculation.

Extensive surveys of large numbers of rabies cases have shown that 10–15% of cases proved positive by mouse inoculation had been missed by direct-smear microscopic examination for Negri bodies. It is therefore strongly recommended that laboratories that provide rabies diagnostic services be equipped to carry out animal inoculation tests on Negri-negative brain tissues, as well as tests such as the FA test.

In the past, the guinea-pig and rabbit were considered the most suitable test animals for this purpose. Since the intracerebral injection of rabies virus into white mice has been shown to produce typical and constant infection, the white mouse is now considered the test animal of preference. The chief advantages of the mouse are the low cost, making it possible to use several animals for one specimen, the relatively short incubation period for street virus, and the consistency of production of Negri bodies in the brains of mice inoculated intracerebrally with street virus. Suckling mice (less than 3 days old) are more susceptible to rabies than adult or weanling mice and should be used wherever possible (see Chapter 6).

A positive microscopic diagnosis is sufficient proof of the presence of rabies. When the microscopic examination proves Negri-negative or questionable, however, samples should be immediately taken from the cerebral cortex, cerebellum and Ammon's horn on each side of the brain, and from the brain stem (medulla). These should be pooled in the emulsifier in preparation for the mouse inoculation test described in Chapter 6. It is important to achieve completely representative sampling of all those parts mentioned on each side for pooling, since there is often great variability in the virus distribution through the brain.

Antimicrobial agents for contaminated and decomposed specimens

It is often difficult to obtain from the field animal brains that are bacteriologically sterile. The head may have been in transit for a long time, or the animal may have been picked up long after death, shot, or clubbed on the head. Also, the cause of death may have been a bacterial encephalitis.

Intracerebral injection of bacteria may cause the death of inoculated mice in 1–3 or more days, before any rabies virus that may be present in the inoculum has had its full incubation period. On the other hand, inoculated mice may live long enough for rabies incubation to be complete, may pass through the typical rabies symptoms of tremors, paralysis and prostration, followed by death, and may show typical Negri bodies and many bacteria in their brain smears.

When bacterial contamination is suspected—for example, if the animal brain is decomposed or when many bacteria are demonstrated on the original brain smears—it is best to treat the brain suspension with an antimicrobial before inoculating it into mice. Of the following agents, penicillin and streptomycin will give the best results. If these antibiotics are not available, any of the other agents may be used.

- *Penicillin and streptomycin.* Add 500 IU of benzylpenicillin sodium and 2 mg (1560 IU) of streptomycin per ml of tissue suspension. Allow to stand for 30 minutes at room temperature before injection. This amount is usually enough, but for very heavily contaminated brains or salivary glands, as many as 1000 IU of penicillin and 3 mg (2340 IU) of streptomycin may be used.

- *Glycerol.* Place the brain specimen in pure glycerol for 48 hours.
- *Phenol 0.5%.* Make up 0.5% phenol in physiological salt solution. This is used as the diluent for making up the 10% tissue inoculum. Hold for 6 hours or overnight. If held for 6 hours, keep the suspension at room temperature. If held for over 6 hours, keep the suspension overnight in the refrigerator.
- *Thiomersal*[1] *1:5000.* Make up a 1:5000 solution of thiomersal in physiological salt solution. This is used as the diluent for making up the 10% tissue inoculum. Hold for 6 hours or overnight. If held for 6 hours, keep the suspension at room temperature. If held for over 6 hours, keep the suspension overnight in the refrigerator.

In order to determine whether contaminating bacteria are present, a portion of all tissue emulsions should be cultured in dextrose infusion broth or similar media, and streaked on a blood-agar plate. The recommended amount is about 0.1 ml of emulsion in 3 ml of broth. The plate should be incubated overnight at 37.5 °C.

Early deaths (1–3 days) among inoculated mice may be attributed to the presence of contaminating bacteria if the cultures show moderate to heavy growth and if many bacteria are found in the brain smears of the dead mice.

Annex

Preparation of Sellers' stain[2]

Examination of slide

In order to save time, the stained slide should initially be studied under low power and areas containing numerous large neurons selected for examination under oil immersion (Figs. 4.6 and 4.7).

Sellers' stain shows the Negri body well differentiated in magenta (heliotrope) to bright red, with well-defined dark-blue to black basophilic inner bodies. All parts of the nerve cell stain blue, and the interstitial tissue stains pink. Erythrocytes stain copper red and can be easily differentiated from the magenta-tinged red of the Negri bodies.

Stock solution

1. Methylene blue	10 g
Methanol (absolute acetone-free)	to make 1000 ml
2. Basic fuchsin	5 g
Methanol (absolute acetone-free)	to make 500 ml

The stock solutions are stored in bottles with screw tops or ground glass stoppers. Certified biological stains are preferable. The dry dyes should preferably contain no less than 85% methylene blue, and no less than 92% basic fuchsin. While absolute acetone-free methanol is recommended, chemically pure (C.P.) methanol meeting American Chemical Society specifications may be substituted if desired.

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[1] Also known as thimerosal and mercurothiolate.

[2] This annex was kindly contributed by the late T. F. Sellers, Former Director Emeritus, Georgia Department of Public Health, Atlanta, GA, USA.

Fig. 4.6 Low-power view of impression, showing field (upper half) rich in neurons for examination under high power

× 200

By courtesy of United States Department of Health, Education and Welfare, Public Health Service, Centers for Disease Control and Prevention (US DHEW-PHS-CDC), Atlanta. GA, USA.

Staining solution

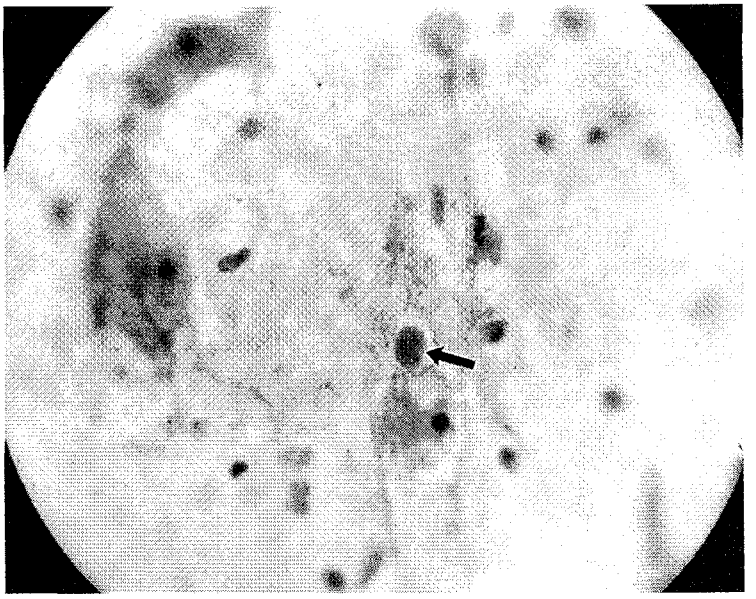
Methylene blue (stock solution No. 1)	2 parts
Basic fuchsin (stock solution No. 2)	1 part

Mix thoroughly but do not filter. Store in a container with a screw cap or ground glass stopper. The staining solution should be left to stand for 24 hours before use, and may be kept indefinitely if protected from evaporation.

Adjustment of stain

When the stock solutions have been accurately prepared, the above proportions will usually produce a stain that will give the desired colour differentiation. However, it is advisable to make a trial stain, and if the results obtained do not equal those illustrated in Plate 5.1,C (page 70), the stain may readily be adjusted. If the stroma is a bright red rather than rose-pink in the thinner areas, and the overall staining effect is reddish, the fuchsin is too dominant. Add methylene blue stock

Fig. 4.7 High-power view of impression, showing Negri bodies



Indicated by arrow.
x 900
By courtesy of US DHEW-PHS-CDC, Atlanta, GA, USA.

solution in measured amounts, checking with a trial stain after each addition until the desired colour balance is obtained. When the methylene blue is too dominant, the Negri bodies are a deep muddy maroon colour and the nerve cells stain too deeply. Adjustment may be made with the fuchsin stock solution in this case.

If the stock solutions are protected from evaporation, more stain may be subsequently prepared using the stock solutions in the adjusted proportions.

Staining procedure

1. Prepare smears or impressions in the usual manner (see page 57); no fixation is required.
2. Immediately, *while the preparation is still moist*, immerse it in the staining solution for 1–5 seconds, depending on the thickness of the smear.
3. Rinse quickly in running tap-water, and air-dry without blotting.
   In some regions tap-water is not satisfactory for rinsing purposes. The suitability of the water may be determined by comparing preparations rinsed with tap-water and others rinsed with distilled water containing 0.66 mol/l phosphate buffer, pH 7.0.

When not in use, the stain must be kept in a tightly closed container to prevent evaporation, which tends to make the fuchsin too dominant. The addition of

absolute methanol will restore the proper balance. It is convenient to keep the staining solution in a screw-capped Coplin jar for daily use. If this is not available, the stain may be stored in a dropper bottle with a ground glass stopper and the smear flooded with the stain. Staining in this manner will not be satisfactory unless the entire process can be completed within a few seconds.

The best results with the stain are obtained when the brain tissue is fresh. If the tissue is decomposed, the characteristic colour differentiation is affected, and although the Negri bodies retain their staining quality, the smear as a whole becomes too red, or at times too blue, and identification of the bodies becomes more difficult.