Tools for safety assessment
Vaccinia-derived recombinant rabies vaccine

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Preface

The vaccinia - rabies glycoprotein (VRG) recombinant virus vaccine (Raboral®) is a live viral vaccine presently used in France, Belgium and in the U.S.A. for the oral vaccination of wild animals against rabies. Target animals for vaccination programmes are the main vectors transmitting the disease: foxes in Europe; raccoons and skunks in the U.S.A. There are plans to use this vaccine in Switzerland; the recombinant viral vaccine would be the first transgenic microorganism released on Swiss soil. The prospect of this release, along with a general public wariness of genetic engineering, has fostered a multitude of on-going discussions dealing with political, ecological, economical, social and ethical issues.

In order to meet the need for readily accessible information, the agency for Biosafety Research and Assessment of Technology Impacts (BATS) of the Swiss Priority Programme Biotechno-logy, funded by the Swiss National Science Foundation, has taken on the task of rigourously reviewing and analysing the available safety-related data concerning VRG. This undertaking was performed in a systematic manner, with emphasis on the phenotype of the transgenic organism rather than on the technique used to produce it. Data concerning possible impact aspects of the environmental release were compiled from existing knowledge of the recombinant virus, VRG; of the inserted glycoprotein G of the ERA rabies strain; of the wild-type vaccinia virus (especially strain Copenhagen, which is the parental strain) and tk^--vaccinia virus (the thymidine kinase gene of vaccinia virus is insertionally inactivated in VRG); and knowledge of (ortho)poxviruses.

It was the aim of this work to make available as much pertinent information as possible related to the safety aspects of VRG. This publication is the assembly of existing data and information into an efficient tool for safety assessment. An extensive list of references is also provided. Both qualitative and quantitative data are presented, and the report attempts to remain impartial regarding sensitive issues where there exist no absolute answers. Some of these more controversial topics include: the probability of VRG persistence in the environment with its related consequences; the overall significance of rare adverse events expected in persons having accidental contact with vaccine fluid. The risks involved with the use of any rabies vaccine, whether recombinant or not, must always be weighed against the health danger arising from uncontrolled rabies for animals and humans.

I. Introduction

I.1. Rabies

Rabies is a dangerous disease known since Babylonian times and is still prevalent in most parts of the world. Two not necessarily related cycles, an urban and a sylvatic one, are responsible for maintaining the rabies epizootic. Urban rabies, which is propagated by affected stray and feral dogs and cats, is the most dangerous to man and accounts for an estimated 99% of all human post-exposure treatments. The WHO figures indicate that 28.000-40.000 persons die from rabies each year, 85 % thereof in Asian countries (WHO, 1993a). In most cases, the rabies virus was transmitted to humans from dogs.

The wild animal species transmitting and maintaining sylvatic rabies varies under specific geographical and ecological conditions and has remained stable over many years. Foxes, skunks and raccoons are the major vectors in North America, dogs in Africa and Asia, and dogs and bats in South America (WHO, 1990). Bats in U.S.A. and South America maintain a classical rabies virus, whereas bats in Europe maintain an independent cycle of the rabies-related European bat lyssavirus.

The present European sylvatic rabies epizootic originated in Poland and has been spreading southwestward since 1939. Although the rabies virus can infect all susceptible mammalian species, both wild and domestic, the red fox (*Vulpes vulpes*) has been the animal vector involved in more than 75% of the cases. The virus is passed onto humans mainly by infected domestic animals. Eradication of rabies from the fox population is expected to drastically reduce the rabies rate in other wildlife or domestic species (Wandeler, 1991; Pastoret *et al.*, 1995).

The rabies virus belongs to the Rhabdoviridae family (Greek *rhabdos*: rod) of enveloped RNA viruses characterised by their shape and the presence of infectious helical nucleocapsids enclosed in a lipid envelope with surface projections. The genome consists of a single molecule of negative single-stranded RNA which is not infectious and is transcribed into five mRNAs, each coding for a single protein (Pastoret *et al.*, 1995). The rabies virus was classified as a class 2 risk group organism by both Störfallverordnung (1992) and Berufsgenossenschaft der Chemischen Industrie (1991) and a class 3 hazard group organism by Grinsted (1995).

Large quantities of virus are shed in the saliva of rabid animals. Biting is the predominant form of disease transmission, and the aggressive behaviour of rabid animals may facilitate transmission. As most infected animals die, it is not possible to establish an immune population. In Europe, the culling of foxes has reduced the number of rabies cases but has not contained the disease. An immune population should be expected to limit the spread of the disease better than a sparse but susceptible population (Wandeler, 1988a and b; Blancou *et al.*, 1986).

The rabies virus initially undergoes rapid replication at the site of inoculation (bite) before resting in the surrounding muscle tissue where it replicates at a low rate. The length of the incubation period varies considerably: among different species, within the same species depending on the inoculation site; and depending on the amount of virus transmitted through the bite. In foxes, incubation periods ranging from 12 days to 15 months have been reported. In humans, the length of the incubation period can range from 4 days up to 19 years before the onset of clinical symptoms, with 30% of all cases reporting onset within 30 days post-infection, 84% of all cases within 90 days and 99% within one year. From the site of inoculation, the virus travels to the central nervous system to proliferate in the brain.

Clinical symptoms of rabies in humans include fever, excitation, dilation of the pupils, excessive salivation, anxiety, fear of swallowing (hydrophobia), uncontrollable spasms of the throat muscles and eventually death from respiratory paralysis. Postexposure treatment of humans to rabies by active and passive immunisation during the incubation period is highly efficient but must be performed before the virus reaches the nervous system whence rabies becomes almost invariably fatal.

Humans and domestic animals are immunised via injection, whereas oral vaccines are used for wild animals. Successful oral immunisation has so far been achieved exclusively by live vaccines such as live attenuated rabies virus vaccines or the vaccinia - rabies glycoprotein recombinant virus vaccine (VRG).

I.2 Vaccines against rabies

I.2.1 Vaccines based on attenuated rabies virus

Wild foxes in Switzerland are currently vaccinated three times a year using baits containing a live but attenuated rabies virus, as part of the Swiss rabies eradication programme (Breitenmoser *et al.*, 1996). The rabies vaccines for oral immunisation of foxes are based on the Street Alabama Dufferin (SAD) strain of rabies virus. Switzerland was the site of the first world-wide field trial with the attenuated strain SAD Berne in 1978 (Steck *et al.*, 1982). Strain SAD B19 was introduced in 1983 in Germany, France and other European countries (Wandeler, 1991). SAD Berne was used in Switzerland for more than 10 years and, during this period, could be reisolated from three animals: a domestic cat, a stone marten and a fox (Wandeler, 1988a; WHO, 1992). These virus samples induced immunity and not disease when injected into healthy animals. Neither laboratory studies nor field experiments have given any indication that the virus could be propagated within a population or community of wild animals (Wandeler, 1988a). In Belgium, field tests using SAD B19 were conducted in September 1986 and in June and September 1987 using tetracycline-marked baits. None of the 216 small mammals (rodents and insectivores) randomly tested was rabies positive but some animals of non target species had attacked the bait as tetracycline could be detected in their jaw bones: 2/51 bank voles (*Clethrionomys glareolus*), 8/71 woodmice (*Apodemus sylvaticus*), 7/18 yellow-necked mice (*Apodemus flavicollis*), 4/24 *Apodemus* sp. and 3/6 stone martens (*Martes foina*) (Brochier *et al.*, 1988a).

Closely monitored vaccination campaigns have resulted in the reduction or elimination of wild rabies in several European countries. However, these attenuated viruses can still be pathogenic for rodents (Pépin, 1985), in particular the closely related strains ERA (Evelyn-Rokitnicki-Abelseth) and SAD (Lawson *et al.*, 1989; Wachendörfer *et al.*, 1978; Winkler *et al.*,1976). In their investigation of the vaccine strains SAD Berne, SAD B19, SAG 1 and VRG, Artois *et al.* (1992) confirmed residual pathogenicity for rodents in both SAD strains, whereas SAG 1 and VRG were found to be innocuous for the three rodent species tested (*Apodemus sp.*, *Arvicola terrestris* and *Clethrionomys glareolus*). It is possible that vaccine-induced mortality for small animals went unnoticed during field trials in Europe because their carcasses would have been either hidden in subterranean burrows or eaten by predators. The SAD virus could also induce fatal disease in domestic animals and wild carnivores with an impaired immune response: for example, distemper in dogs (Wandeler *et al.*, 1988b). Moreover, these strains harbor pathogenic potential for humans. Humans exposed to SAD-derived attenuated strains of rabies virus must be treated with a conventional inactivated rabies vaccine, which elicits good cross-protective immunity (WHO, 1993; BAG, 1993). In the Swiss rabies vaccination programme, strain SAD Berne was replaced by the strain SAG 1 in 1991. The vaccine strain SAG 1 was derived from the SAD Berne strain by a mutation in Arg 333 of the G protein which resulted in an attenuated pathogenicity. The vaccine strain presently used in Switzerland is strain SAG 2 derived from SAD Berne by

double mutation of the codon 333, making SAG 2 genetically more stable than strain SAG 1 (Masson *et al.*, 1994).

I.2.2 Vaccine based on vaccinia virus

The vaccinia - rabies glycoprotein (VRG) recombinant virus vaccine (Raboral®) is a live viral vaccine containing 10^8 pfu per dose. It is constructed by insertion of the DNA copy coding for glycoprotein G of the ERA rabies virus strain into the thymidine kinase (tk) locus of the Copenhagen strain of vaccinia virus using homologous recombination (Kieny *et al.*, 1984). Poxviruses have been chosen as ideal vectors for the expression of foreign genes. Not only do poxviruses accommodate insertion of large amounts of foreign genetic information, but they also express large quantities of glycosylated proteins and stimulate humoral as well as cell-mediated immunity. Vaccinia virus currently is the major vehicle for constructing subunit vaccines (Quinnan, 1985; Mackett, 1990 and 1992; Tartaglia *et al.*, 1990; Binns *et al.*, 1992).

Raboral® (Rhône Mérieux, France) has been introduced and studied in extensive field tests in Belgium and France (Pastoret *et al.*, 1995). In Belgium, a total of 250 chicken head baits containing VRG was distributed over an isolated area of 6 km^2 in October 1987. By day 15, 63 % of the bait had been taken up with 4 out of 145 trapped rodents having attacked the bait (Pastoret *et al.*, 1992a). Another documented large-scale field trial covering 435 km^2 was conducted in 1988 in the province of Luxembourg (South Belgium). The vaccine suspension was contained in a plastic sachet concealed in a bait made of fishmeal and fishoil. Field tests were also conducted in November 1989, April 1990 and October 1990 over 2'200 km^2 in Belgium for a total of 25.000 baits distributed from the air. Of the 188 foxes analysed during this time, 74% to 81% of the adult foxes and 49% of the young foxes had taken up the vaccine (Pastoret *et al.*, 1992b). In France, two distributions of VRG contained in a machine-made bait were carried out in October 1988 and May 1989. The test area of 53 km^2 was divided into a control area (174 baits without VRG) and an experimental area (187 baits with VRG). Since then, several campaigns using the VRG vaccine have been carried out with machine-made baits dropped by helicopters over 10.000 km^2 for a mean bait density of 15/km^2 (Pastoret, 1995). From 1988 to 1996 a cumulative quantity of 7.476.414 Raboral® doses have been used in Europe (Stöhr WHO, 1997).

Whereas the red fox (*Vulpes vulpes*) is the main vector of rabies transmission in Europe, the striped skunk (*Mephitis mephitis*) and the raccoon (*Procyon lotor*) are the predominant vectors in the U.S.A.. VRG in machine-made baits produced by Wistar (U.S.A.) was successfully tested in raccoons (Koprowski, 1989; Rupprecht *et al.*, 1993; Rupprecht *et al.*, 1986). Field tests using this vaccine in the USA were performed in Virginia in 1990 and Pennsylvania in 1991 (Rupprecht *et al.*, 1992a).

Once the VRG vaccine is ingested by the fox, the vaccinia virus begins to replicate and express rabies glycoprotein G, which stimulates the host organism to develop humoral and cellular immune responses to the rabies glycoprotein. Although VRG cannot cause rabies, high levels of neutralising antibodies against the rabies virus are rapidly produced in the immunised animal, with protection of the animal extending also against several street rabies viruses (Pastoret *et al.*, 1992a). Immunity lasts a minimum of 12 months in cubs and 18 months in adult animals (Brochier *et al.*, 1990; Desmettre *et al.*, 1990).

VRG bait per se is not more efficient than attenuated rabies virus vaccines. In a comparative trial, 7 out of 15 foxes resisted challenge after immunisation with strain SAD B19, 5 out of 9 foxes with strain SAG 1, and 6 out of 10 foxes after immunisation with the recombinant vaccine VRG. All 6 control animals from this trial died after challenge with the rabies virus (Aubert, 1996). However, the greater reported efficacy of VRG from French field tests could be the direct result of its higher heat stability (Aubert *et al.* 1994).

The heat stability of VRG is therefore the main motivation for using this vaccine in Switzerland. In addition to the normal yearly vaccination schedule, additional campaigns could be realised during the end of May and early June when it is still possible to vaccinate young foxes at their dens. Because of a marked decrease of rabies in 1995, the introduction of Raboral® in northwestern Switzerland was not necessary in 1996.

I.3 Scope and structure of this report

This report focuses mainly on the ecological and human health aspects brought about by the release of the recombinant rabies vaccine (VRG) into the environment. The methodology can be divided into three key steps as described in Figure 1. In the first step, possible impact aspects (concerns) are identified based on: regulatory guidelines, characteristics of the recombinant organism, literature on the safety of recombinant vaccines (Hahn, 1992; Guillaume *et al.*, 1991; Baxby, 1991; Collier, 1991; WHO, 1990; Kaplan, 1989) and literature on ecological aspects of environmental release (Tiedje *et al.*, 1989). A broad overview of the existing scientific information is provided in a second step (part II). Using data obtained from the literature, an assessment of the impact aspects is performed in a third step (part III) with conventional rabies virus based vaccine as a point of comparison. A standardisation scheme for assessing possible impacts will also be presented.

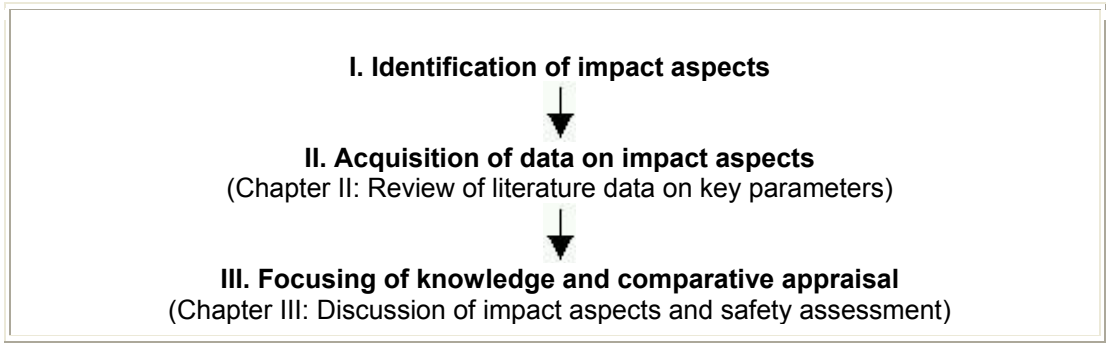

Fig. 1: Key steps in a safety assessment.

The literature review is a vital element of this report (part II). The information available on the recombinant organism and its biological elements of construction provides a firm basis for judging impact aspects related to release. Raboral® contains live vaccinia rabies glycoprotein recombinant viruses (VRG). VRG is constructed from the following elements, which are key parameters for a potential hazard assessment:

1. **recipient / parental or host organism**: vaccinia, strain Copenhagen
   locus: thymidine kinase

2. **donor organism**: rabies virus, ERA strain
3. **insert**: glycoprotein G

Taking into consideration the possible recombination of VRG with other (pox-)viruses after release into the environment, another key parameter must be introduced:

4. **viruses potentially derived from recombination** of VRG in the field

As Table 1 illustrates, the review of literature data is structured according to these key parameters. Empirical and experimental knowledge is summarised and extended according to the principles of familiarity: through knowledge of different strains of vaccinia virus and through analogies with other poxviruses. For the parameter **1** , no data are available and information concerning ecology and recombination is extrapolated from data on poxviruses.

Table 1: Topics related to key parameters and structure of the literature review (part II).

Topic:	Chapter:
1 Vaccinia Strain Copenhagen tk^- phenotype	II.1
Poxviruses	II.2
2 Rabies glycoprotein G	II.3
3 Vaccinia rabies glycoprotein recombinant virus (VRG)	II.4
4 Viruses potentially derived from **recombination** of VRG in the field	II.5

I.4 General remarks

Environmentally released transgenic organisms could have subtle and long-term effects on biological communities and natural ecosystems (Bruggemann, 1993). There is a potential for transgenic organisms to establish themselves in the environment as persistent populations. There is also a risk that they might introduce transgenes into existing populations through introgression or other means. Organisms containing new combinations of genetic information might also play novel ecological roles (Tiedje, 1989).

Strategies for designing recombinant and any live viral vaccine share common concerns for issues such as lack of attenuation, reversion to virulence, oncogenesis, and recombination (Hahn, 1992). General concerns about genetic engineering are not discussed in this report because: 'The phenotype of the transgenic organism, not the technique used to produce it, is the appropriate focus of ecological risk assessment and regulatory oversight' (Tiedje *et al.*, 1989). A thorough evaluation of the VRG vaccine must examine the vaccinia virus: its pathogenicity, host range and ecology (Czerny, 1996; Baxby, 1986; Brown, 1986; Kaplan, 1989).

Vaccinia has a broad host range, and unlike some other members of the orthopoxvirus family, is not indigenous to European wild life so far. Once released it

might persist in the environment with unpredictable fate and effects. The wide dissemination of vaccinia-based vaccine resulting from extensive use increases the probability of vaccinia recombination with other orthopoxviruses present in target and nontarget animals. The vaccinia virus has been used most successfully for the vaccination against smallpox in humans, but has, in rare instances, caused serious adverse effects.

Before launching any type of vaccination programme against sylvatic rabies, thorough studies on the chosen vaccine (recombinant or live attenuated virus) efficacy as well as on its cost/risk analysis are necessary. The cyclic nature of rabies epizootics makes comparisons of efficacy drawn from field studies a difficult task (Anderson, 1991; Brochier *et al.*, 1990; Brochier *et al.*, 1991). It is not within the scope of this report to evaluate efficacy and cost/risk factors.

Criticism against the wildlife rabies vaccination programme exists (Mc Nally, 1994; Bruggemann, 1993), however, European and North American authorities and most virologists plead for this preventive measure.

Finally, before releasing any genetically modified organism into the environment, economic, social and ethical concerns also should be carefully addressed.

II. Review of literature data on key parameters

II.1 Vaccinia

The vaccinia virus is a class 2 risk group organism belonging to the family of poxviruses (chapter II.2), which comprises the largest and most complex animal viruses known (Störfallverordnung, 1992; Berufsgenossenschaft der chemischen Industrie, 1991). Vaccinia virus was used as an immunising agent against human smallpox during the global eradication programme which sought to vaccinate the population (mostly children) via intradermal (scarification) inoculation of vaccinia virus.

The vaccinia virus is a double stranded DNA-virus, whose minus strand is transcribed into mRNA. Transcription of vaccinia virus is characterised by a cascade of events: early gene transcription requiring early promotor elements, intermediate gene transcription requiring the presence of two intermediate gene transcription factors synthesised early in infection, and late gene transcription requiring three intermediate gene products. DNA replication itself does not appear to require specific origins of replication since any DNA transfected into vaccinia virus infected cells is replicated (Wittek, 1994). Replication of the vaccinia virus takes place in the cytoplasm of the infected host cell (Fenner *et al.*, 1989).

II.1.1 Strains and genetic stability

Genomic differences among vaccinia virus strains are mainly due to variability in the terminal regions of the DNA molecule. The central part of the genome is highly conserved. Studies conducted in certain tissue culture cell lines have demonstrated that functions coded in the DNA from the left-hand and right-hand terminal regions of the orthopoxvirus genome are not essential for replication of the virus, but rather determine host range and virulence (Buller *et al.*, 1985a).

A variety of mutant vaccinia virus strains have been isolated, which often display altered characteristics compared to the wild-type strain. Most strains of vaccinia virus produce red haemorrhagic lesions (pocks) on the chorioallantoic membrane of chicken embryos. White pock lesions, however, arise with a relatively high frequency; the viruses isolated from such pocks have been designated white pock mutants. Some white pock mutants were also associated with an altered host range. Other mutants which are temperature-sensitive or drug-resistant have also been isolated (Wittek, 1994).

In vaccinia viruses isolated from cases of postvaccinial encephalitis, deviations in plaque-morphology, heat sensitivity and growth characteristics in cell cultures relative to the vaccine strain have been observed (Ehrengut *et al.*, 1975).

The terms 'dermal vaccinia' and 'neurovaccinia' are mentioned in the early literature on the subject and refer to the method used to propagate the virus. In dermal vaccinia, the virus was maintained by passage through animals inoculated by scarification. Neurovaccinia refers to viral propagation by intracerebral inoculation of rabbits, a method particularly used between 1920 and 1940. These 'neurovaccinia virus' strains produced encephalitis in rabbits. Some of these strains were capable of establishing enzootic disease causing substantial mortality in rabbit colonies and were subsequently called 'rabbitpox' (Fenner *et al.*, 1989). DNA mapping has revealed that neurovaccinia (rabbitpox) and dermovaccina strains have very similar DNA maps (Binns *et al.*, 1992; Wittek *et al.*, 1977).

Variations in poxvirus characteristics are not necessarily due to variations in the genotype. Some host cell material is always incorporated during the packaging and assembly of the progeny virus and may be responsible for variations in phenotypic characteristics of viruses with identical genotype.

The parental strain used for the construction of the vaccinia-rabies glycoprotein (VRG) virus is the Copenhagen strain of vaccinia virus (Kieny *et al.*, 1984), whose genome has been entirely sequenced (Goebel *et al.*, 1990). This strain has been used as a smallpox vaccine but was associated with more side effects, particularly neurological complications. In a comparative clinical study with four vaccine strains used during the smallpox eradication programme, the Copenhagen strain was found to be more pathogenic than strains Berne, Elstree (Lister) and Equador (Polak, 1963). Therefore, in 1963, the less pathogenic Elstree vaccinia strain replaced the Copenhagen strain in the RIV (Rijks Instituut voor de Volksgezondheid, Netherlands) smallpox vaccine (Polak,1972).

Karupiah *et al.* (1990) showed that several strains of vaccinia virus, after intravenous or intra-peritoneal inoculation, but not after subcutaneous inoculation in the foot pad, replicated in the ovaries of normal inbred mice and caused sufficient damage to ovarian follicles to decrease fertility of the mice. The mouse-adapted vaccinia virus strain WR had a greater affinity for growth in ovaries than the vaccine strains Elstree, NYBH or Copenhagen, although strain Copenhagen also was observed to decrease fertility of infected mice. A thymidine kinase negative vaccinia virus tested was also able to replicate in ovarian tissue of mice.

II.1.2 Thymidine kinase-negative vaccinia virus

In the VRG virus, the thymidine kinase gene of the parental vaccinia virus has been insertionally inactivated. The role of the virus-coded thymidine kinase (TK) is to provide a sufficient pool of thymidine triphosphate for virus DNA synthesis in host cells which are not actively dividing and, therefore, have lower endogenous levels of precursors for DNA synthesis (Buller *et al.*, 1985a).

Thymidine kinase-negative (tk^-) vaccinia virus recombinants have been shown to be less pathogenic for mice, rabbits and chimpanzees than their respective wild-type viruses expressing thymidine kinase (Buller *et al*., 1992). The results of Buller *et al.* (1985b) suggest that the decreased pathogenicity of tk^- recombinant virus comes from its decreased ability to disseminate to and/or replicate in the internal organs and brain.

Whether inactivation of wild-type vaccinia thymidine kinase locus also decreases virulence in immunosupressed mice is controversial. Koprowski (1989) reported a decreased virulence in immunosuppressed mice and in Swiss nude mice after thymidine kinase inactivation. Rodriguez *et al.* (1989) denied an effect by thymidine kinase inactivation in the case of immunodepressed mice but reported a vaccinia recombinant with a left-end deletion and alterations in the 14-kD fusion protein encoding gene to be about 100 times less virulent in immunosupressed animals than a wild-type recombinant with a thymidine kinase negative phenotype.

It is generally accepted that tk^- vaccinia is less virulent in humans, although caution is advisable in extrapolating data on the observed attenuating effects from animal models to humans (Baxby, 1991).

II.1.3 Persistence and spread

II.1.3.1 Origin

The origin of vaccinia virus is unknown. Five hypotheses (Fenner, 1992; Behbehani, 1983) suggest vaccinia virus to be:

1. derived from variola virus by passage in cows
2. derived from variola virus by passage in humans (variolation)
3. derived through hybridisation between cowpox and variola viruses
4. a fossil virus maintained in the laboratory but otherwise extinct
5. derived from cowpox virus by repeated passage on the skin of animals

None of these hypotheses has conclusively been proven.

II.1.3.2 Host range

The vaccinia virus has an extremely broad host range comprising mammals and birds. It is considered a 'laboratory virus' with no known natural host and has not yet spread within the European wildlife.

Host range may not be a stable trait, since selection of variants with a broader host range was observed after repeated passages of a cloned vaccinia virus stock (Dales *et al.*, 1981).

II.1.3.3 Tissue tropism and persistence in the host

The usual pathological manifestation of the vaccinia virus is either a localised skin lesion or a generalised rash, depending on the host species infected as well as on the viral strain. Clinical data on the pathology of vaccinia is available from the smallpox eradication programme during which the vaccine was administered to participants intradermally (chapter II.1.4.1). Normally, no viraemia was observed post-administration, and the vaccinia virus remained localised in the scab and in the nearest lymph nodes. However, postvaccinial complications including generalised vaccinia, were occasionally observed. Progressive vaccinia occurred among persons with cellular immunodeficiency. Persons with atopic eczema or a history of atopic eczema were more susceptible to developing eczema vaccinatum. This condition was also observed in unvaccinated members of this risk group after viral transmission had occurred from vaccinated contact persons.

The ability to spread within the host organism may be altered according to the method used to cultivate and maintain the vaccinia virus (chapter II.1.1). Dermal vaccinia produces localised pustular skin lesions in man, cow and rabbit and a generalised pustular rash in water buffaloes; generalised pustular rash in man from dermal vaccinia has rarely been observed. The neurovaccinia (rabbitpox) virus produces a generalised pustular rash in rabbits (Fenner *et al.*, 1989).

Tissue tropism depends on the viral strain as well as on the method of inoculation. As mentioned earlier (chapter II.1.1), the intravenous or intraperitoneal injection, but not the subcutaneous injection, of the Copenhagen strain of vaccinia results in viral replication with damage to murine ovaries (Karupiah *et al.*, 1990). Certain strains of vaccinia virus proliferate in nervous tissue, but the types of cells involved have not been identified (Behbehani. 1983). After rabbitpox virus infection of rabbits by the respiratory route, virus could always be isolated from the lungs; very high titers of virus were found in the gonads and adrenal glands (Fenner *et al.*, 1989).

Buller *et al.* (1991) claim that the vaccinia virus cannot be conserved in a latent state within the animal host, as the poxvirus genome is apparently unstable in a host cell. The relatively large size of poxviruses encourages their clearance by phagocytic cells of the reticuloendothelial system. Moreover, the early modification of the plasma membrane of the host cell with virus-specified polypeptides provides the means for antibody-dependent cell-mediated cytotoxicity (ADCC) or cytotoxic T lymphocytes (CTL) killing of infected cells prior to the production of the progeny virus.

Although most investigators have failed to demonstrate persistent infection with orthopoxviruses, there are several reports on the isolation of orthopoxviruses from tissues of experimentally infected animals inoculated several weeks earlier but appearing healthy at the time of viral isolation (see also chapter II.2.3.2). The neurovaccinia virus could be reisolated from the spleen and testis of rabbits after 114 and 133 days post-intradermal adminstration. In mice pre-treated with cyclophosphamide, vaccinia virus could be reisolated (after cocultivation only) 60 days after inoculation (reviewed by Fenner *et al.*, 1989; Binns *et al.*, 1992).

II.1.3.4 Transmission

Suspected portals of entry are the respiratory tract and skin for the neurovaccinia (rabbitpox) virus in rabbits and for the vaccinia virus in (especially young) water buffaloes (Fenner *et al.*, 1989). Other strains of vaccinia virus may also infect rabbits through their respiratory tract, but the viruses do not replicate to high titres outside the lungs. There is little information available for other than laboratory animals on the pathogenicity of the vaccinia virus after its infection of the respiratory tract. No firm evidence for aerosol transmission in humans was found in the very extensive studies on the complications associated with smallpox vaccination.

Few data are available about shedding of vaccinia virus in faeces (a known phenomenon for other poxviruses such as ectromelia virus of the orthopox virus genus), respiratory secretions or from skin lesions. However, it has been observed that maximum viral shedding occurs 4-14 days following intradermal vaccination and that virus can be isolated from the site of vaccination until the scab separates from the skin (Centers for Disease Control, 1991).

Human to human transmission

During the global smallpox eradication programme in the 1960s, an enormous number of children were intradermally vaccinated using vaccinia virus. Transmission from vaccinated persons to unvaccinated contact persons, usually in a family setting, was known to occur. The overall incidence rate of transmission to contacts was 27 infections per million total vaccinations (Centers for Disease Control, 1991); transmission was not a common event. Unvaccinated infants with (a history of) atopic eczema were more susceptible for receiving the virus through transmission.

Occasionally, conjunctival inoculation of contacts could occur when an individual (usually a child) inadvertently rubbed an eye after touching a weeping vaccinial vesicle on the skin of a vaccinated child or adult (Levine, 1995).

In an isolated case it was documented that mites had acted as viral shuttles in transmitting vaccinia from one person to another (Blaskovic *et al.*, 1967).

Human to animal transmission

There is documented evidence for vaccinia transmission between humans and animals, and the literature has been reviewed by Fenner *et al.* (1989) and Kaplan (1989). It has been shown possible to isolate the vaccinia virus from domestic animals that had been in contact with recently vaccinated humans. In the past, vaccinia virus has also spread from vaccinated humans to domestic animals such as cows (through milking), camels and buffaloes, before spreading within the herd (WHO, 1990). Sometimes calves were infected, but usually the virus did not persist within the herd. The only example of continued transmission within a herd of animals without contact with recently vaccinated humans was buffalopox in parts of India. During outbreaks of buffalopox, unvaccinated humans have become infected by the virus, which could be differentiated from the strains of vaccinia virus used in India (WHO, 1990). There is evidence that the enzootic buffalopox in Indian water buffaloes was caused by vaccinia variants in India (Baxby *et al.*, 1971; Lal *et al.*, 1977) as well as in Egypt (Fenner *et al.*, 1989). The genomic organisation of the

buffalopox virus isolated from animals in India was shown to be similar to that of the vaccinia virus (Fenner *et al.*, 1989). As the origin of both buffalopox and vaccinia is unknown, it is not possible to prove conclusively, whether vaccinia was the source of buffalopox (Yilma, 1995). The same can be said for the rabbitpox virus derived from naturally infected rabbits which could have originated from the infection of domestic rabbits after contact with vaccinated humans (Fenner *et al.*, 1989).

II.1.4 Pathogenicity and virulence

The global smallpox vaccination programme is the source of most knowledge about human infection with the vaccinia virus. Various strains of the vaccinia virus were administered intra-dermally. As mentioned earlier (chapter II.1.1), pathogenicity and virulence varies with different viral strains. Moreover, pathogenicity of the same viral strain may vary with different host strains, different cultivation methods or different routes of infection. More detailed information on vaccinia virus virulence and interactions with the host are given in the review by Buller *et al.* (1992).

II.1.4.1 Adverse reactions observed after vaccination

Vaccination of individuals with the vaccinia virus sometimes provoked complications such as: inadvertent auto- or hetero-inoculation, generalised vaccinia, eczema vaccinatum, progressive vaccinia and postvaccinial encephalitis. The frequency of adverse reactions to vaccination varied with the type of vaccine virus used, the age of the vaccinee, and his or her state of health. It has been estimated that after primary vaccination, the risk of death was about 1 per million, the risk of hospitalisation with encephalitis, eczema vaccinatum or progressive vaccinia was about 10 per million, and the risk of other serious complications including eczema vaccinatum, accidental ocular inoculation of vaccinia, or superinfection of a variety of skin conditions approached 1,000 per million (Behbehani, 1983).

The rate of viral transmission from vaccinated to unvaccinated persons was given as 27 infections per million total vaccinations (Centers for Disease Control, 1991). Over 60% of contact transmission resulted in uncomplicated inadvertent inoculation, whereas approximately 30% of contact transmission resulted in eczema vaccinatum, which sometimes was fatal.

Inadvertent auto- or heteroinoculation

Inadvertent auto-inoculation from the inoculation site to other body sites was the most frequent complication of vaccinia administration and accounts for about half of all complications observed (Centers for Disease Control, 1991). The most common body sites involved were the face, eyelids, nose, mouth, genitalia, and rectum. Most lesions healed without specific therapy, but in some cases keratitis or scarring of the cornea impaired vision or even caused loss of the eye.

Generalised vaccinia

Generalised vaccinia was characterised by a vesicular rash of varying extent induced after a systemic infection with a brief viraemia. There was no obvious reason - such as immunodeficiency - to account for the generalisation. The rash was generally self-limiting and required little or no therapy except among patients with critical conditions

or serious underlying illnesses. The incidence rate of generalised vaccinia was 23.4 cases per million vaccinations (Centers for Disease Control, 1991).

Progressive vaccinia

Progressive vaccinia (vaccinia necrosum, vaccinia gangrenosa) is a severe, potentially fatal illness characterised by progressive necrosis at the vaccination site, often leading to metastatic lesions. It was observed almost exclusively among immunodeficient persons. Treatment with vaccinia-immunoglobulin was rarely effective.

The figures published are 0.8 (Centers for Disease Control, 1991) and 1.6 cases of progressive vaccinia per million vaccinations, with a case fatality rate of almost 90% (Kaplan, 1989).

Neurological symptoms

Neurological complications were the most serious health complications arising from vaccination and often are referred to as 'postvaccinial encephalitis'. Kaplan (1989) differentiated between postvaccinial encephalopathy and postvaccinial encephalitis. **Postvaccinial encephalopathy** was primarily diagnosed in patients less than 2 years old (98% of the cases), with more than 50% of the affected patients dying on the first day of onset. Incomplete recovery was common, with patients sustaining residual paralysis and cerebral damage. **Postvaccinial encephalitis** or encephalomyelitis, in contrast, occurred in patients aged over 2 years. The case fatality rate was about 35% within a week of onset and recovery was generally complete.

Both syndromes were difficult to diagnose and quantify due to a lack of international standardisation of the criteria for diagnosis and due to possible confusion with coincidental encephalopathy / encephalitis. The highest frequency for both complications was 60 per million primary vaccinations (WHO, 1990), whereas for Wyeth strain, USA, a number of 2.9 cases of postvaccinal encephalitis per million vaccinations was recorded.

As strain Copenhagen, which is VRG's parental strain, was associated with higher levels of side effects, particularly neurological complications (chapter II.1.1), in 1963, the Dutch authorities have disallowed the future use of this strain as the standard vaccine strain in Holland (Polak, 1972).

Neurological symptoms were predominantly associated with the primary vaccination. Incidence and outcome of these complications were unpredictable and no risk groups could be identified. The relationship between incidence and age of the vaccinees is not entirely clear. Although primary vaccinees less than one year of age were reported to be the most frequently affected (Centers for Disease Control, 1991), especially in some of the countries reporting a large incidence of cases, it was clearly shown that risk increased with increasing age at primary vaccination (Kaplan, 1989). The incidence rate of postvaccinial encephalitis in adults was considerably higher than in children (Blaskovic *et al.*, 1967; Kaplan, 1989). For the immunisation of vaccinia-negative adults, it was recommended as a precaution, to either pre-treat these patients with inactivated vaccinia antigen or to combine the vaccination with a

simultaneous treatment with vaccinia immunoglobulin (Mohr *et al.*, 1975). The foregoing figures were based on observations during the vaccination programme against smallpox, where mainly infants were vaccinated.

Etiology and pathogenesis of postvaccinial encephalitis are still unclear. Pathological changes characteristic of postvaccinial encephalitis seem to be closely related to the presence of the viral antigens, but isolation of vaccinia virus from brains of deceased patients was not always successful. Angulo (1964) was able to isolate vaccinia virus from the brain of a young man who had died from postvaccinial meningo-encephalitis. Histological findings in this case were comparable with those of experimental allergic encephalitis. Kurata *et al.* (1977) investigated five lethal cases of postvaccinial encephalitis. Virus isolation on chick embryo chorioallantoic membrane was negative in all cases. In 3 out of the 5 cases, however, vaccinia antigen was detected either with or without sodium thiocyanate treatment to dissociate the immunoglobulins from the antigen-antibody complex. Gurvich *et al.* (1983) isolated vaccinia virus from blood, cerebrospinal fluid and pharyngeal secretions of 23 out of 40 children suffering from postvaccinial encephalitis. Positive isolations were found until the 34th day after vaccination from blood, until the 32nd day from cerebrospinal fluid and until the 35th day from the pharynx. Collier (1991) has summarised these observations and concluded, that postvaccinial encephalomyelitis might be more closely related to an abnormal host response, possibly mediated by immunological factors, rather than to the strain or type of virus involved. This conclusion would be consistent with the finding, that the incidence in adults, who are more allergy-prone, was much higher than in children.

II.1.4.2 Therapy

Two therapeutic agents, anti-vaccinia immunoglobulin and N-methylisatin thiosemicarbazone (Marboran) have been used to reduce the severity of certain adverse reactions related to vaccination. Administering anti-vaccinia immunoglobulin notably diminished the case fatality of eczema vaccinatum. Treatment with anti-vaccinia-immunoglobulin, however, has often been ineffective against progressive vaccinia. Whether or not immunoglobulin treatment is effective against postvaccinial encephalitis is still controversial. According to WHO (1990) figures, immunoglobulin treatment was not effective against postvaccinial encephalitis, however, simultaneous treatment of vaccinees at higher risk (patients with allergies, adult primary vaccinees, and in cases of long intervals before revaccination) with human anti-vaccinia immunoglobulins was beneficial (Mohr *et al.*, 1975). Controlled clinical studies to determine whether Marboran has a beneficial effect on vaccinia necrosum have not been undertaken (WHO, 1990).

II.1.4.3 Risk groups

Pregnant women

There have been rare cases of foetal vaccinia syndrome acquired through maternal vaccination. Two of the documented cases of foetal vaccinia syndrome were the result of vaccinia virus transmission from recently vaccinated children to pregnant women (Levine, 1995). Adverse effects (pregnancy disturbances or, more rarely, miscarriage) in pregnant women from vaccination was 47 % for those vaccinated

between week 4 and 5 of pregnancy; or 18 out of 67 pregnant women vaccinated during the first trimester (Blaskovic *et al.*, 1967).

Immunodeficient hosts

Immunodeficient pesons (diagnosed as having neoplastic diseases such as leukemia and lymphoma, generalised malignancy, agammaglobulinemia, defective cell mediated immunity; or undergoing therapy with alkylating agents, antimetabolites, radiation, or large doses of corticosteroids) form a high risk group for immunisation using the vaccinia virus. Not only is there possible enhancement of vaccinia virus replication in these patients, but there is also a likelihood of severe post-vaccination complications, especially in HIV-positive patients. Redfield *et al.* (1987) reported the case of an apparently healthy young recruit who became ill after multiple immunisations, one of which was against smallpox. Early in the complex clinical development of his disease, he developed widespread generalised vaccinia which responded to treatment with vaccinia immunoglobulin, but died 20 months after the onset, with a firm diagnosis of AIDS. It is still controversial whether immunisation with vaccinia may transform an asymptomatic HIV-infection into a symptomatic one (Kaplan, 1989).

A recent report suggests that two HIV-infected persons may have died of a progressive vaccinia-like illness after treatment with inactivated autologous lymphocytes infected with a recombinant HIV-vaccinia virus (Guillaume *et al.*, 1991). To date, progressive vaccinia has occurred only in persons with immunodeficiencies.

Infants with (a history of) atopic eczema

In the 1960s, vaccinia was administered routinely to healthy infants at 12 months of age in the US and in Europe. Although children with eczema were not directly vaccinated, they were still at risk for contact transmission of vaccinia virus from other, recently vaccinated children. Exposed infants with a compromised protective integumental barrier often developed severe eczema vaccinatum syndrome. Clinical symptoms were vaccinia infection of the entire eczematous areas of the skin, accompanied by high fever and toxaemia. Even after vaccinia immunoglobulin became available as a therapy, eczema vaccinatum remained a sometimes fatal complication not only in vaccinated children but also in unvaccinated children exposed to virus through transmission.

II.1.4.4 Impact on the human immune system

Poxviruses are relatively successful animal pathogens despite effective animal host defense mechanisms (chapter II.1.3.3). This success is due in part to subtle viral strategies such as immunosuppression (leporipoxviruses) or anti-interferon properties (vaccinia virus). A growing list of those virus-encoded functions interacting with the host's defence is being elucidated (Buller *et al.*, 1991 and 1992).

SERPINs belong to a superfamily of related proteins critical for the regulation of serine proteases, which exert control over a variety of events associated with connective tissue turnover, coagulation, fibrinolysis, complement activation, and inflammatory reactions. Genes coding for proteins with amino acid sequences closely related to SERPINs have been identified in vaccinia. One simple hypothesis claims

that one of these proteins might hinder the generation of host chemotactic signals and would thus delay the onset or decrease the magnitude of the inflammatory response, therefore permitting more extensive virus replication and spread within the host (Buller *et al.*, 1991).

The vaccinia virus complement-binding protein (VCBP) has been shown to block the antibody-mediated classical complement cascade, which may explain the relative unimportance of antibody in the recovery from poxvirus infections.

II.2 Other poxviruses

II.2.1 Classification of poxviruses

Poxviruses are classified into two subfamilies: chordopoxvirinae (poxviruses of the vertebrates) and entomopoxvirinae (poxviruses of the insects) (Bergoin *et al.*, 1971). As shown in Table 2, the subfamily of chordopoxvirinae can further be subdivided into 8 different genera. Vaccinia virus is the prototype virus of the orthopoxvirus genus. The major antigens of the orthopoxviruses are unrelated to those from the other poxvirus genera. Within one genus, however, there is little antigenic diversity. Historically, members of one genus were differentiated primarily by their pathological effects in a variety of laboratory animals and cell culture systems (Dumbell, 1985). The classification of the entomopoxvirinae will not be discussed in this report. There is no evidence that any member of the chordopoxvirinae subfamily replicates in invertebrate hosts, although invertebrates sometimes act as shuttle vectors for the transmission of chordopoxvirinae as was shown for mosquitoes (myxoma virus) and in one single case for mites (vaccinia virus) (II.2.3.3). There is no serological crossreactivity between the subfamilies (Dales and Pogo, 1981).

Table 2: *Classification of Poxviridae* (**SUBFAMILY, genus**, species, prototype virus).

CHORDOPOXVIRINAE:

Orthopoxviruses:

Buffalopox, California vole pox, camelpox, cowpox, ectromelia, monkeypox, rabbitpox, raccoon pox, tatera pox, Uasin Gishu pox, vaccinia, variola and vole pox viruses

Parapoxviruses:

Chamois contagious ecthyma, Orf, pseudocowpox, stomatitis papulosa viruses

Avipoxviruses

Canary pox, fowlpox, junco pox, pigeon pox, quail pox, sparrow pox, starling pox, turkey pox

Capripoxviruses:

Goat pox, sheep pox, lumpy skin disease viruses

Leporipoxviruses:

Hare fibroma, myxoma, rabbit (Shope) fibroma, squirrel fibroma viruses

Suipoxviruses:

Swinepoxvirus

Molluscipoxviruses:

Molluscum contagiosum virus

Yatapoxvirus:

Yaba and Tanapoxviruses

ENTOMOPOXVIRINAE

II.2.2 Genetic stability

Poxviruses exhibit genetic variability with known spontaneous alterations in cytopathology and virulence (Ball, 1987; Pickup *et al.*, 1984). Defined vaccinia virus stocks exist having viral particles of different structure and infectivity, as reflected by heterogenicity in lengths of inverted terminal repeat segments (Robert Koch-Institut des Bundesgesundheitsamtes, 1993).

Considerable variation in the terminal genome structure among different poxvirus species has been observed. Spontaneous recombinants in the terminal sequences of the genome of individual poxvirus species have been isolated, indicating a strategy for expanding the poxvirus genome and thereby allowing for viral evolution or acquisition of novel functions (see also II.5.1).

II.2.3 Persistence and spread

II.2.3.1 Host ranges, reservoirs and distribution

There is a possibility that released recombinant vaccinia-rabies viruses might persist and spread within wild animal populations. The likelihood and the consequences of such an event must be considered. This will be done by examining the ecology of poxviruses in general, with special attention to the ecology of members of the orthopoxvirus genus. The vast host range of the vaccinia virus overlaps with the host ranges of cowpox virus and some other members of the orthopoxviruses. This observation is important, because the recombination of two viruses is possible only when they share the same host cell (chapter II.5).

In Table 3, chordopoxvirinae are listed in alphabetical order with details on their hazard group, pathogenicity to humans, experimental host range, natural animal sources, reservoir and geographic range.

Reservoirs of orthopoxviruses other than vaccinia virus are mainly wild rodents or other small mammals (Arita and Gromyko, 1982; Baxby, 1977; Marennikova *et al.*, 1977).

In Turkmenia, different species of wild rodents were tested for the presence of antibodies against cowpox virus. Anti-cowpox antibodies were detected in four species of wild rodents, especially in gerbils and susliks.

Orthopoxvirus antibodies could be detected in 2 out of 28 woodmice and 7 out of 20 short tailed voles trapped in Southern England and Wales (Kaplan *et al.*, 1980). The exact species of orthopoxvirus was not determined (Fenner *et al.*, 1989). In a study of German cats, 2 - 10 % of those tested had antibodies against orthopoxvirus (Czerny *et al.*, 1994). Because the reisolated viruses from these cats exhibited low infectivity, the authors suggested that small rodents rather than other cats (horizontal transmission) were responsible for the spread of orthopoxvirus to cats.

Of 703 blood samples from wild red foxes (*Vulpes vulpes*) in Germany, 6.5% tested positive for the presence of antibodies against orthopoxviruses (Henning *et al.*, 1995). The study was conducted over 10 districts of the federal state of Brandenburg and the sampling was considered representative for an area of about 5000 km^2. The

percentage of seropositive animals ranged from 2.8% (of 108 sera tested) in the district of Wittstock to 9.9 % (of 192 sera tested) in the district of Neuruppin and was particularly high in three smaller areas with a topography characterised by rivers, brooks and lakes. The natural host reservoir of these orthopoxviruses, later identified as cowpox virus (Czerny, 1996: personal communication), is therefore expected to be found among animals adapted to this environment.

In 1989, **buffalopox** virus was isolated from animals in India and shown to have a genomic organisation similar to vaccinia virus (Fenner *et al.*, 1989). It is generally assumed that buffalopox is a variant of the vaccinia virus transmitted from vaccinated humans to water buffaloes (Baxby *et al.*, 1971; Lal *et al.*, 1977; WHO, 1990; Yilma, 1995).

Rabbitpox virus, synonymous to neurovaccinia virus (chapter II.1.1), is transmitted via the respiratory tract and has caused outbreaks of a lethal pox disease in colonies of laboratory rabbits in Utrecht and New York (Wittek, 1994).

Cowpox is a rare disease found only in Britain and Western Europe. Human cowpox usually causes localised skin lesions often accompanied by fever, local edema, lymphangitis and lymphadenitis (Fenner *et al.*, 1989). The source of human infection for 13 out of 16 cases reported from 1969 to 1981 remained unknown. There was significant disease in some of these 16 cases, particularly in young children. Although humans have generally acquired cowpox from infected cattle, the disease can also come from other sources. Both cows and humans are only sporadic indicator hosts of the cowpox virus, whereas rodents are suspected to be the natural host reservoirs. Cowpox may also be maintained in apparently healthy small wild animals. The cowpox virus has a wide host range including felines (see 'catpox'). Whereas domestic cats develop minor disease from cowpox virus, larger felines (zoo animals) develop a fatal fulminating pulmonary infection (Marennikova *et al.*, 1977).

Catpox usually results in skin lesions without any other clinical symptoms in cats and was first observed in England (Thomsett *et al.* 1978; Bennett *et al.*, 1986). It has long been suspected that cowpox and catpox were the same virus (Martin *et al.*, 1984), and recent results confirm this theory (Czerny, 1996: personal communication). There are increased incidences of catpox transmission from domestic cats to humans (Robert Koch- Institut des Bundesgesundheitsamtes, 1993). In 1991, a lethal case of transmission of a virus similar to cowpox from cat to an immunosuppressed man was reported (Czerny *et al.*, 1991).

Smallpox, the disease brought about by the **variola** virus, has been causing deadly epidemics for thousands of years. Variolation, the immunisation of persons with material taken from typical smallpox lesions, was practiced in Asia and Africa for many centuries. Data collected in Britain between 1723 and 1727 demonstrated the benefit of variolation in disease prevention. The observed death rate from variolation was between one death in 48 cases and one death in 60 cases, whereas death from natural disease was one in 6 cases. Edward Jenner (1749-1823) was the first physician to pioneer the preventive vaccination of individuals with the less pathogenic cowpox virus in place of variolation. Jenner's hope for eradicating smallpox in humans remained virtually ignored until 1950, when the WHO-approved eradication programme using the vaccinia virus in a mass vaccination strategy began in the

Americas. In 1977, the last person to acquire naturally occuring smallpox in the world recovered from this disease in Somalia (Behbehani, 1983).

The success of the WHO smallpox eradication programme was made possible only because of the fact that the variola virus has no known animal reservoir. Investigation of poxvirus hosts and reservoirs and isolation of new viruses has therefore intensified in recent years (for review see Baxby, 1977).

The clinical symptoms of human infection with the **monkeypox** virus are indistinguishable from those of smallpox; the overall case fatality rate in unvaccinated persons from monkeypox is about 11.2%. Severity of illness is higher in children than in adults, and the case fatality rate for children under 4 years of age is 15%. Monkeypox is a rare zoonosis which occurs in tropical rain forest areas of west and central Africa. Seroepidemiological surveys suggest that forest-dwelling monkeys, squirrels, porcupines, or pangolins may be involved in the natural cycle of transmission. Between September 1970 and May 1971, six cases of human infection with monkeypox virus were identified in three west African countries (Lourie *et al.*, 1972). The mode of transmission to humans, however, is not understood yet. Because human-to-human transmission of monkeypox is low, minor epidemiological significance is currently given to this virus.

In one documented case, a poxvirus was isolated from a wild gerbil (*Tatera kempii*) caught in northern Dahomey, Africa, at the time of an epidemic of human smallpox. When inoculated into a rhesus monkey, this poxvirus caused fever but no skin eruption while inducing seroconversion and protection from subsequent challenge with monkeypox virus (Lourie *et al.*, 1975).

Whitepox virus (in African monkeys and rodents) is similar to the smallpox virus. The first two of the so-called whitepox viruses were isolated from monkey cell cultures in 1964; four more viruses were isolated from the kidney tissues of a chimpanzee, a sala monkey and two African rodents obtained in the wild between 1971 and 1975 in Zaire (Behbehani, 1983). As a contamination of these cell cultures by variola virus could not be entirely excluded, there were no efforts to further classify the whitepox virus.

II.2.3.2 Tissue tropism and persistence in the host

Orthopoxviruses spread in the host's body through the lymphatic system and bloodstream as cell-associated virions, although some infectivity can also be found free in plasma. Using tissue cultures, at least three host range genes have been identified as important for in vitro replication of poxviruses in various cell types. These genes may also be important for tissue tropism in vivo (Buller *et al.*, 1992).

It has been possible to isolate orthopoxviruses from apparently healthy animals experimentally infected some weeks earlier (Fenner *et al.*, 1989). Cowpox virus was isolated from kidneys and lungs of cotton rats and rats up to 6 weeks after intranasal inoculation; monkeypox virus was isolated from kidneys and lungs of cotton rats and rats up to 6 weeks after intracardiac injection, ectromelia virus could be isolated from lungs and spleen of two of 114 mice tested for five weeks and more after recovery from infection. Big gerbils and yellow susliks were infected with the Turkmenia strain of cowpox virus in an experimental setting. These animals sustained an ensuing

severe disease with high mortality. In the surviving animals, this cowpox strain could be isolated from the kidneys and testes for up to 5 weeks.

II.2.3.3 Transmission

There are various possible routes for poxviruses to enter their host during natural infection: through the respiratory tract (aerosols), through the skin (small abrasions, arthropods) or through the digestive tract. Fenner *et al.* (1989) have established a list of suspected portals of entry used by various orthopoxviruses which cause systemic disease.

The **respiratory tract** has been the suspected portal of entry for all orthopoxviruses and host species mentioned: camelpox virus in camels, cowpox virus in rodents and felines, ectromelia virus in mice, monkeypoxvirus in man, monkeys and squirrels, vaccinia virus in rabbits and water buffaloes, and variola virus in man and monkeys. Rabbits could be infected with aerosols composed of dermal strains of vaccinia virus or neurovaccinia (rabbitpox) virus. Rabbitpox transmission occurred over distances of up to 12 feet (Fenner *et al.*, 1989). No evidence for aerosol transmission, however, was found either in the extensive studies on the complications associated with smallpox vaccination using the vaccinia virus or in epidemiological studies of human cowpox infections.

The **digestive tract** has been the suspected portal of entry for cowpox virus, ectromelia virus and monkeypox virus. Mice could be systematically infected by the oral route when large doses of ectromelia virus were given; about 20% were infected when low doses of virus were used.

Although there were often no visible signs of infection in mice after administration of small amounts of virus, the ectromelia virus could nevertheless be isolated for several weeks post-infection from the faeces, the intestinal tract, and lesions at the base of the tail near the anus (Fenner *et al.*, 1989).

The mite *Ornithonyssus bacoti* may act as a vector of ectromelia virus, a non-tumourigenic virus. These mites are contaminated when they ingest the blood from viraemic mice; they then continue the transmission cycle when eaten by healthy mice.

Ectromelia virus, vaccinia virus and variola virus may enter their hosts through small abrasions in the **skin**.

Arthropod vectors play a key role in the dermal transmission of tumourigenic orthopoxviruses in wild animals (myxoma virus, rabbit (Shope) fibroma virus, squirrel fibroma virus and hare fibroma virus) and in domestic pigs (swinepox virus) (Mc Fadden, 1994). It is known that myxoma virus and rabbit Shope fibroma virus are spread within the rabbit population by arthropod vectors, notably mosquitoes (Fenner *et al.*, 1965, Myers, 1954; Dalmat, 1959). During the campaigns conducted for controlling the rabbit population, myxoma virus was deliberately released in Australia (1950) and in Europe (1952). It was observed during these campaigns that arthropod vectors (mosquitoes and bugs) helped to spread the virus. The rabbit (Shope) fibroma virus was found to be extremely stable in several species of mosquitoes, a

bedbug and a mite and was able to maintain its infectious capacity for long periods of time (Dalmat, 1959).

For the non-tumourigenic viruses such as vaccinia virus, ectromelia virus and variola virus, there is no proof of disease transmission by arthropods. As mentioned earlier (II.1.3.4.), there has only been one known case of mites transmitting vaccinia virus from one person to another.

II.2.4 Pathogenicity and virulence

A comprehensive review on poxvirus pathogenesis has been written by Buller *et al.* (1991). Their review covers aspects such as poxvirus entry, replication, and interactions with the host, as well as host responses to infection.

The pathogenesis of a poxvirus infection is either restricted to the site of dermal infection (localised) or generalised throughout the host organism. For localised infections, a broad spectrum of host reactivity has been observed: none to a limited immune response as in the molluscum contagiosum infection of the human epidermis; or an early granulomatous response followed by T- and B-cell responses in the Shope fibroma virus infection of rabbit skin. When viral infection causes generalised symptoms in the host, as with ectromelia in mice and variola virus in humans, a primary viraemia may be followed by secondary one and accompanied by erythematous rash. The same poxvirus may provoke both localised and generalised disease, but only in different hosts.

Some poxviruses induce benign tumours, such as leporipoxviruses (myxoma virus, hare fibroma virus, rabbit (Shope) fibroma virus, squirrel fibroma virus) and related poxviruses such as molluscum contagiosum virus (human), swinepoxvirus (pig), tanapox virus (human) and Yaba virus (monkeys).

As discussed by Fenner *et al.* (1989), different species of orthopoxviruses may exhibit different degrees of pathogenicity in the same host. In adult mice, for example, infection by ectromelia virus was highly lethal; infection by vaccinia virus was highly to very highly lethal, depending on viral strains; and infection with monkeypox virus and raccoon poxvirus were highly lethal. In baby mice lethality was high after infection with vole poxvirus, variable after infection with cowpox virus and low after infections with camelpox virus, tatera poxvirus and variola virus. Monkeys also developed varying degrees of disease severity after infection with orthopoxviruses. A generalised rash was induced by infection with monkeypox virus and variola virus, whereas localised, large dermal lesions were induced by camelpox virus, cowpox virus and vaccinia virus. Furthermore, monkeys were susceptible but exhibited no rash after infection with Uasin Gishu poxvirus.

The same virus can cause different symptoms in different host species. In tapeti (wild rabbit), myxoma virus produces localised benign fibromas, whereas infected domestic rabbits develop a highly lethal generalised infection. In an outbreak of a pox disease within carnivora of the Moscow Zoo, the infectious agent was shown to be very closely related to cowpox virus (Marennikova et al., 1977). Two forms of the disease were found: a fatal, fulminant pulmonary form without skin lesions and a dermal form with rash. The pulmonary form of infection was observed in all of the lions and cheetahs and in one black panther. The symptoms of the disease were:

rejection of food, lethargy, fever, frequent breathing, paroxysmal cough and cyanosis of mucous membranes and, ultimately, death. The dermal form of the Moscow poxvirus disease was lethal in anteaters and a female ocelot, but pumas, jaguars one black panther and Asian cats recovered after refusal of food for one or two days and after they had developed scabs and alopezia.

Bedson and Dumbell (1964) showed that, out of several hybrids between rabbitpox virus (pathogenic for rabbits and mice) and variola minor virus (pathogenic for man), some were pathogenic for rabbits, others for mice. Because in these recombination experiments the pathogenicity for two different host species was found to segregate so easily, Dumbell warned against casual extrapolation of pathogenicity results from animals to man (Kaplan, 1989; Dumbell, 1985).

II.3 Rabies glycoprotein G

The glycoprotein G constitutes surface projections embedded in the rabies virus envelope. It is the sole viral protein capable of inducing synthesis of virus-neutralising antibodies in the host (Blancou *et al.*, 1986), and it stimulates cellular immunity. In a rabies virus infection, the glycoprotein G is involved in several key steps of the viral replication cycle. With the aid of this protein, the virus adheres to the host cell, thus determining cell tropism, then fuses with the membrane of endocytic vesicles which allows the nucleocapsid to penetrate into the cytoplasm. Later in the replication cycle, glycoprotein G also plays a role in the budding of viral particles (Tuffereau *et al.*, 1989). Glycoprotein G at least partially determines the pathogenicity of the rabies virus, since the introduction of a mutation at arginine 333 of this protein (selection of mutants resisting neutralisation by appropriate monoclonal antibodies) renders the virus avirulent for mice and some other species (Coulon, 1983; Tuffereau, 1989).

There is no evidence for the incorporation of the rabies glycoprotein G into the envelope of VRG viruses. It is generally assumed that the rabies glycoprotein G is synthesised in the host, but not incorporated into the envelope of progeny viruses. The safety of the recombinant VRG virus hinges on this observation, because incorporation of the glycoprotein G into the envelope might alter the tissue tropism of the VRG virus as compared to the vaccinia virus. During animal safety tests with VRG (see chapter II.4.2.2 for more details) no VRG virus was detectable in the brain of vaccinated animals (Pastoret *et al.*, 1995).

II.4 Vaccinia - rabies glycoprotein recombinant virus (VRG)

II.4.1 Genetic stability

The genetic stability of the recombinant construct was checked throughout 10 passages in vero cell (the cell line routinely used to propagate the virus) culture, and throughout 7 passages in mice following footpad or intracerebral inoculation. Desmettre *et al.* (1990) concluded that no evidence could be found for genomic breakdown or for change in biological properties of the recombinant virus.

In a first series of experiments designed to test for a possible reversion to virulence, 6-week-old mice were inoculated with VRG through the plantar pads of their posterior feet. After 48 hours the mice were killed and the top ends of their feet were homogenised in a physiological solution. The homogenised extract was then clarified

and the supernatant used to reinoculate other mice by the intraplantar method. After four serial subpassages of the VRG virus using the same method, virus could no longer be isolated from the homogenised tissues. In a second series of experiments, six-week-old mice were inoculated intracerebrally with VRG virus. The brains of mice which died 3-4 days following inoculation were collected, homogenised and subpassaged a second time into other mice by the intracerebral route. The inoculum from the second passage of virus did not cause mortalities, and the mice were sacrificed six days after inoculation for the collection of brain material for a third and fourth subpassage. Virus could no longer be isolated after the third and fourth blind passage through mice brains (Glosser, 1989).

VRG viruses reisolated from infected raccoons were shown to have retained their recombinant characteristics using the method of staining fixed virus-infected cells with a fluorescent antibody (polyclonal anti-ERA rabies glycoprotein conjugate) technique (Glosser, 1989).

II.4.2 Persistence and spread

II.4.2.1 Persistence in the environment

VRG vaccine in distributed baits was shown to have a very high durability in the environment. In field tests with recombinant VRG virus, the first significant decrease of virus titer was detected only after 4 months (Brochier *et al.*, 1990). Although durability increases the probability for successfully vaccinating the target animal population, it also implies a prolonged overall availability of the infectious virus strain after the release. Since an animal host is necessary for the virus to replicate, persistence in hosts and host reservoirs for the VRG virus are key points to consider in the estimation of the overall persistence of this virus in the ecosystem.

II.4.2.2 Tissue tropism and persistence in target animals

Data are available on the detection of VRG virus in different animal organs after oral infection and on the time scale of recovery. A proper assessment of these parameters is necessary for determining the probability of viral transmission in the field.

Parental vaccinia virus and VRG were compared for their preferred multiplication site in vaccinated foxes. Techniques such as virus isolation, titration, indirect immunofluorescence, and polymerase chain reaction (PCR) for specific viral DNA detection were used to investigate for presence of the viruses in several fox organs.

26 foxes were fed with 10^8 TCID50 (Tissue Culture Infecting Dose) of either VRG or vaccinia virus strain Copenhagen, then were killed at 12, 24, 48 and 96 hours after inoculation (Thomas *et al.*, 1990). Both viruses could be isolated from the tonsils of some of the foxes during the first 48 h after inoculation at titres between 10^2 and $10^{4.3}$ TCID50/ml. Whereas indirect immunofluorescence confirmed the presence of the VRG virus only in the tonsils of some of the foxes, PCR allowed the detection of VRG in the tonsils of both of two foxes tested 24 hours after infection and in all three foxes tested 48 hours after infection. Using PCR, it was found that the buccal mucosa (one of two foxes tested positive at 48 hours after infection) and the soft palate (one of two foxes tested positive at 24 hours after infection; one of three foxes tested positive at

48 hours after infection) also harboured VRG virus. The vaccinia virus was detected in the tonsils (one fox tested at 48 hours after infection) and in the buccal mucosa (one fox tested at 24 hours after infection; one fox tested at 48 hours after infection) of foxes.

No VRG virus was detected in serum, faeces nor in any of the other organs tested (brain, spleen, parotid glands, maxillary glands, liver, retropharyngeal lymph nodes, submaxillary lymph nodes, and mesenteric lymph nodes) of the vaccinated foxes. The detection limit was $10^{1.5}$ TCID50/ml. In addition, VRG could not be isolated from foxes inoculated with viral stock passaged once in foxes (Thomas *et al.*, 1990).

Results of other unpublished experiments demonstrate that performing tonsillectomy on the foxes did not affect the immunity conferred by VRG; viraemia was not observed on days 0, 2, 3, 4, 5, 6, 7, 8, and 14 in inoculated foxes. Boulanger (1995) examined oropharingeal swabs of foxes and was able to reisolate VRG from tonsils of one out of two foxes the day after oral inoculation. Pastoret *et al.* (1995) concluded that orally administered recombinant virus tended to multiply locally at low levels; they also emphasised that neither vaccinia nor VRG virus could be detected in the brain.

Raccoons were given VRG orally. On days 1, 2, 3, 5, 9, 11, and 14 after inoculation, the following tissues from these animals were obtained for virus isolation: brainstem, buccal mucosa, tongue, lung, stomach, tonsil, esophagus, salivary glands, parotid/submandular lymph nodes, thymus, trachea, duodenum, liver, spleen, kidney, urinary bladder, blood, and cerebrospinal fluid. Viral presence was found in tonsils and parotid/submandibular lymph nodes on days 1 and 2; in buccal mucosa on day 2 (Rupprecht *et al.*, 1988). A more detailed discussion on studies performed in raccoons and skunks is given elsewhere (Pastoret *et al.*, 1992a).

II.4.2.3 Bait uptake by nontarget animals

Bait uptake monitoring using tetracycline markers (included within the bait), performed after field trials and vaccination campaigns, revealed that wild boars (S*us scrofa*) and domestic carnivores had also ingested the vaccine baits. Moreover, a significant proportion of the baits had partly been eaten by small mammals, which appear to be the group of animals playing the role of a host reservoir for poxviruses. It was observed that baits were also consumed by nontarget organisms such as snails and birds (Artois *et al.,* 1990). In a Belgian field trial, a significant proportion of baits was partially eaten by rodents and insects (*Necrophorus verspillo* and *Geotrupes* sp.) (Brochier *et al.*, 1990). The jaws of 222 wild animals from 19 species were inspected post-mortem for the presence of tetracycline. 16 of 26 foxes, 3 of 7 stone martens (*Martes foina*), 3 of 11 domestic or feral cats (*Felis catus*), 5 of 50 wood mice (*Apodemus* sp.), 15 of 32 wild boars (*Sus scrofa*), and 1 of 10 carrion crows (*Corvus corone*) had tetracycline in their jaws (Brochier *et al.*, 1990). It should be mentioned, however, that tetracycline was included only in the bait material surrounding the vaccine-containing sachet, but not in the vaccine fluid itself. Therefore, it is difficult to precisely determine the number of animals having taken up the VRG vaccine from the number having simply attacked the bait without ingesting the VRG virus.

II.4.2.4 Transmission

The available literature on the transmissibility and on the possible routes of infection of vaccinia virus and vaccinia-derived recombinant viruses fails to reach consensus. It is generally assumed, however, that transmission of VRG will be a rare event. Glosser (1992) largely dismissed the likelihood of direct biologial transmission via viral shedding based on the results of contact experiments with rodents, badgers, cats, cattle, ferrets, and foxes. Furthermore, he emphasised that transmission of the VRG virus in the laboratory (by inoculation of organ extracts from infected laboratory animals to healthy animals) was limited to two or three passages.

Nevertheless, transmission of the VRG virus from one animal to another cannot be totally excluded. Conceivable routes of infection, apart from direct contact with the bait are: contact transmission between animals living together, of the opposite sex, or female-offspring pairs; food chain contamination (infected small mammals) for carnivores; or possibly transmission through biting.

In a study by Pastoret *et al.* (1992a), no VRG virus could be detected in the salivary glands (parotid and maxillar glands) and faeces of vaccinated foxes. This result led the authors to conclude that the likelihood of viral transmission was low. In earlier experiments designed to test for potential contagiousness of VRG, unvaccinated foxes were held in close contact with vaccinated ones (Blancou *et al.*, 1986; Brochier *et al.*, 1988b). Seroconversion was not observed in these contact animals and no transmission of immunising amounts of VRG occurred. There was, however, one exception of successful viral transmission through reciprocal biting between a male vaccinated fox and a control female fox a few minutes after oral vaccination of the male (Blancou *et al.*, 1986). This unvaccinated female fox developed significant levels of rabies neutralising antibodies and successfully resisted severe challenge infection.

Contact transmission experiments were also performed with other animal species such as rodents, badgers, cats, cattle, and ferrets. Contact animals not directly inoculated or fed with VRG, but held in the same containment room with vaccinated animals, did not mount an immune response to the virus and remained fully susceptible to challenge with a virulent street rabies virus. The results were in keeping with the results of contact experiments in foxes. In general, no case of transmission by excretion of the vaccinal strain has been reported (OECD, 1995).

Evidence for contact transmission has been cited, however, in pair-bonded raccoons of the opposite sex (2 out of 5 male-female pairs tested) and in lactating female-offspring pairs (Glosser, 1989). In their study on the efficacy of VRG in raccoons, Rupprecht *et al.* (1988) investigated the effects of VRG in utero and upon suckling animals. Two adult female raccoons that were immunised intramuscularly with 1.0 ml (10^7 pfu (plaque forming units) /ml) of VRG within 30 days before parturition gave birth to healthy litters of three to four young animals. At birth, all littermates had anti-rabies antibodies at levels comparable to those of their vaccinated mothers. Since no virus could be isolated from these offsprings, it was assumed that passive transfer of maternal antibody had taken place, as opposed to active infection in utero. In another experiment, the same authors exposed three suckling raccoons (3-4 weeks old) to their mother immediately after she had received 1.0 ml of VRG (10^7 pfu/ml) orally. All offsprings remained healthy, seroconverted within 28 days, and survived a peripheral

rabies virus challenge. The actual route of transmission in this latter case (via lactation or offspring grooming) was not documented.

There has been one reported, controversial case on the possible transmission of VRG virus between humans and animals. This case has caused considerable political and scientific debate (Crawford, 1986; Fox, 1987b). In 1986, the Pan American Health Organisation, aided by the Philadelphia-based Wistar Institute, tested the recombinant vaccinia-rabies vaccine on cows at an experimental farm in Argentina. The study was interrupted and all experimental animals killed when Argentinian authorities, who had not been previously informed of the experimental undertaking, discovered the unauthorised study. According to a ministry of public health report, serum samples from the control animals taken before they were killed indicated the presence of anti-rabies antibodies which had not been present in these animals before the experiment. In addition, "some" of the 17 people exposed in varying degrees to the vaccine-inoculated animals also had developed anti-rabies antibodies, without any ensuing health problems (Palca, 1988). Preliminary results, obtained by scientists at the National Institute of Microbiology of Argentina, indicated that all vaccinated and contact animals had seroconverted 6 months after the beginning of the experiment (Sineriz, 1988). These data were asserted but not documented. The unpublished report from the commission appointed by the Argentinian government to handle this case stated that there were no apparent public health consequences engendered from the banned test. To further appease public opinion, the Wistar Institute, where the vaccine had been developed, claimed that none of their employees and none of their unvaccinated control animals exposed to vaccinated animals had ever developed an increased rabies antibody titre (Fox, 1987a).

Other animal-to-animal viral transmission studies have been conducted with recombinant vaccinia (Wyeth strain) vaccine against rinderpest. There was no observed horizontal viral transmission from vaccinated to unvaccinated contact animals. Furthermore, all animals, whether vaccinated or not, were free of pock lesions (Yilma, 1995).

II.4.3 Pathogenicity and virulence

The innocuity of both the parental vaccinia virus (Strain Copenhagen) and the recombinant VRG virus in foxes has been tested using intradermal, subcutaneous and oral administration routes (Blancou *et al*., 1986). Mild, localised inflammation was observed at the sites of injection, with spontaneous regression within 8 days. The intensity of the cutaneous reaction was more pronounced with the vaccinia virus than with the VRG virus. No clinical signs or lesions were observed in the inoculated foxes at 1, 6, 12 and 18 months after vaccination with VRG. This absence of pathogenicity was observed independent of the dose of inoculation (10^2 to 10^{10} TCID50) and of the inoculation route (oral, intramuscular, intraduodenal, subcutaneous, intradermic, ocular, and intranasal) (Pastoret, 1992a).

Other nontarget laboratory and domestic animals that have been vaccinated with VRG and subseqently monitored for vaccine-related adverse effects include: laboratory mice (oral, intradermal, intramuscular), hamsters (intramuscular), rabbits (oral, intradermal, intramuscular), ferrets (oral), cattle (intradermal, subcutaneous, intramuscular), sheep (oral), pigs (oral, intramuscular), dogs (oral, subcutaneous),

and cats (oral, subcutaneous) (Pastoret *et al.*, 1992a). All animals were observed for a period of 28 days or more. Except for the development of typical pox virus lesions at the intradermal inoculation site, all other inoculation methods produced neither local nor generalised reactions (clinical symptom or lesion). In keeping with the observed lack of horizontal VRG transmission in foxes (see chapter II.4.2.4), unvaccinated contact dogs, cats, cattle, and ferrets also tested negative for the presence of anti-rabies antibodies.

Nude mice, used as a model for immunosuppressed animals, did not develop any local or general reaction following inoculation into the footpad or by the intraperitoneal route (Desmettre *et al.*, 1990, Pastoret *et al.*, 1992a).

Several European nontarget wild animal species, chosen due to their opportunistic feeding behaviour and their presence in the areas where the vaccine was to be distributed, have also been tested for vaccine safety (Pastoret, 1995). These animals include: Daubenton's bat (*Myotis daubentoni*), wild boar (*Sus scrofa*), Eurasian badger (*Meles meles*), wood mouse (*Apodemus sylvaticus*), yellow-necked mouse (*Apodemus flavicollis*), bank vole (*Clethrionomys glareolus*), common vole (*Microtus arvalis*), field vole (*Microtus agrestis*), water vole (*Arvicola terrestris*), common buzzard (*Buteo buteo*), kestrel (*Falco tinnunculus*), carrion crow (*Corvus corone*), magpie (*Pica pica*) and jay (*Garrulus glandarius*). Prior to vaccination, all animals were verified as serologically negative for rabies virus-neutralising antibodies. At 28 days after oral vaccination, rabies virus antibody titers greater than 0.5 I.U./ml appeared in 2 of 12 Daubenton's bats, 2 of 4 wild boars, 2 of 6 Eurasian badgers, 16 of 27 wood mice, 5 of 7 yellow-necked mice, 3 of 4 *Apodemu*s sp., 8 of 13 bank voles, 1 of 1 field vole, but in none of the birds. This evidence for an effective immune response suggested that VRG was able to multiply in these animals (Brochier *et al.*, 1989a). Daily observations for a minimum of 28 days after inoculation and postmortem examination conducted on all animals did not result in the detection of pox lesions in the oral mucosa of mammals, on the skin of mammals, and on the unfeathered portions of birds. All animals remained asymptomatic and no deaths were observed for at least 28 days post-vaccination (Brochier *et al.*, 1989a; Pastoret *et al.*, 1992a and b).

Only 50% of the badgers tested in this study resisted challenge infection with rabies virus, indicating a very weak efficacy of VRG vaccine in badgers as compared to other species.

In order to assess primate responses to VRG, squirrel monkeys were inoculated by intradermal scarification with either vaccinia (strains Copenhagen and New York Board of Health) or VRG virus and chimpanzees were orally vaccinated with 10^9 pfu of VRG. Neither the squirrel monkeys nor the chimpanzees developed any clinical symptoms other than the usual lesion at the vaccination site. A total of 8 of 11 chimpanzees and 7 of 8 squirrel monkeys vaccinated with VRG developed rabies-virus neutralising antibodies (Rupprecht *et al.*, 1992b).

No data exist on the potential pathogenicity of VRG in humans. There is, however, one documented case of an accidental human infection with a recombinant vaccinia virus derived from the WR strain of vaccinia virus (expressing the vesicular stomatitis virus serotype Indiana N protein). This person, who had been vaccinated against smallpox some 30 years earlier, inadvertently inoculated himself through a small cut

on a finger with the recombinant vaccinia strain. He developed very mild symptoms with lesions completely healing by day 25 post-infection (Jones *et al.*, 1986).

In 1988 (October 24 to November 1988) a first field test with VRG was carried out in Belgium (province de Luxembourg) covering 435 km^2 of a region characterised by various habitats, 25 km^2 thereof being intensively controlled. More than 220 animals of 19 species were collected and inspected postmortem for pox-like lesions. After necropsy, the following organs were removed for further inspection: brain for rabies diagnosis, jaw for tetracycline (tracer for bait uptake) detection and blood for the determination of antibodies against vaccinia and rabies. No pox-like lesions were found in these animals and no abnormal morbidity or mortality in wild and domestic animals was observed during the experiment and the next 7 months of close monitoring (Brochier *et al.*, 1990).

II.4.4 Asymptomatic carriers of rabies

There is a theoretical possibility for the emergence of asymptomatic carriers of wild rabies virus, occurring when naturally infected animals are vaccinated during the incubation period. This has to be taken into consideration for any type of rabies vaccine - attenuated or recombinant. The influence of vaccination with VRG on the onset of the rabies disease in infected foxes and on the delay before death from rabies has been investigated in a laboratory setting (Brochier *et al.*, 1989b). All infected foxes, whether vaccinated or not, died from rabies. Animals which were vaccinated early after exposure to the rabies virus died quicker than unvaccinated controls. In contrast, animals vaccinated later in the rabies incubation period died after the unvaccinated controls. These results suggest that VRG can influence the pathogenesis of rabies infection in foxes. Brochier *et al.* (1989b) deduced from these investigations that both "early" and "late" death phenomena might occur in animals incubating rabies, but they did not believe that asymptomatic carriers of wild rabies virus would emerge after vaccination campaigns.

II.4.5. Efficacy of vaccinia based recombinant vaccines in vaccinia-positive individuals

The efficacy of recombinant vaccinia virus vaccines might conceivably be decreased by pre-existing immunity to vaccinia virus or to another recombinant vaccinia virus. A series of recombinant vaccinia viruses exist for the expression of foreign viral proteins such as rabies virus glycoprotein G, hepatitis B surface antigen, herpes simplex virus glycoprotein D, Epstein Barr virus glycoprotein, influenza virus haemagglutinin, vesicular stomatitis virus nucleoprotein, human respiratory syncytial virus proteins, HIV envelope proteins, and cytomegalovirus proteins. Booster 'responses' with the same vaccine (expressing an identical pattern of proteins) are usually excellent as was, for example, documented with the VRG vaccine in rabbits (Wiktor *et al.*, 1985). Vaccination using a different recombinant vaccine based on vaccinia virus, however, is expected to be more efficacious in vaccinia-naive individuals than in vaccinia-primed individuals. When vaccinia-primed individuals were vaccinated at a later data with a different recombinant vaccinia-based vaccine expressing new antigen, these individuals were found to have only some (decreased) T-cell response but no or minor serological response.

Cooney *et al.* (1991) vaccinated healthy volunteers with recombinant vaccinia virus expressing the gp 160 envelope protein of HIV. Persons having been previously vaccinated against smallpox showed transient HIV-specific T-cell responses, but no specific antibody response. By contrast, vaccinia-naive volunteers showed good T-cell and serological responses to the HIV envelope protein.

Openshaw *et al.* (1991) have reported the consequences of an accidental human infection with recombinant vaccinia viruses expressing two individual proteins from respiratory syncytial virus. The laboratory technician involved had been vaccinated against smallpox in 1989. The reaction following this vaccination had been strong, including local swelling, an erythematous papular rash across the trunk, and severe regional lymphadenopathy. These systemic symptoms, obliging the technician to be absent from work, had resolved without any specific medical intervention. After the technician's infection accident with the recombinant vaccinia viruses, however, he had only local effects at the sites of inoculation and repeated tests for respiratory syncytial virus-specific IgG, IgM, IgA, and IgA were all negative. Other tests showed, that T-cells from the patient were able to recognise the 22 kD protein of respiratory syncytial virus.

Experiments with multiple vaccinia vaccinations in mice have been reported by Andrew *et al.* (1992). Mice were initially immunised with vaccinia virus and reimmunised 4 weeks later with a recombinant vaccinia virus expressing influenza haemagglutinin (VV-HA). Only some of these vaccinia-primed mice had developed titers of specific antibody for HA above the detection level, with all titers of HA-specific antibodies lower than the titers observed in vaccinia-naive control mice. Consequently, vaccinia-primed mice showed higher mortality and morbidity upon challenge with influenza virus compared to vaccinia-naive mice. The titer of HA-specific antibody and the level of protection were observed to be inversely proportional to the anti-vaccinia titer at the time of immunisation with VV-HA.

II.5. Viruses potentially derived from recombination of VRG in the field

Recombination between two viruses (either by homologous recombination or by direct ligation of viral genomic DNA) can occur only if both viruses infect the same cell. Therefore the host ranges and the tissue tropisms of the viruses in question must overlap.

Whereas poxviruses recombine easily with other members of the same genus, their recombination with viruses belonging to other families is not very likely. Because virus-specific promoter sequences are required for transcription, the effective transfer of genetic material from a poxvirus is expected to take place only if the recipient organism is another poxvirus, preferably of the same genus. Moreover, poxviruses, African swine fever virus, and to some extent iridoviruses are the only DNA viruses which replicate in the cytoplasm of the infected host cell (Fenner *et al.*, 1989).

II.5.1 Recombination within poxvirus family: possible outcome

Vaccinia virus has an extremely broad host range and the release of vaccinia-derived VRG could lead to viral hybridisations with indigenous poxviruses present in a susceptible wild species (Table 3) (Kaplan, 1989; Baxby *et al.*, 1986). Although the likelihood of such a recombination event under natural conditions is generally

considered to be low, with the likelihood of a recombination generating a more virulent progeny virus considered even lower, recombinations, representing a "hypothetical risk" (WHO, 1993b), still have to be taken into consideration.

In general, recombination was found to occur with high frequency in poxvirus-infected cells (Moss, 1992; Ball, 1987; Evans *et al.*, 1988). Among different poxvirus species, considerable variation in the terminal genome structure was observed. Moreover, spontaneous recombinants in the terminal sequences of the genome of individual poxvirus species were isolated, and homologous recombination in vaccinia virus DNA seems to be a frequent event.

The molecular mechanisms involved in recombination are not well understood. Repetitive sequence elements provide substantial targets for homologous DNA recombination (Ball, 1987; Spyropoulos, 1988). Homologous recombination requires early gene products, whereas resolution of concatamer junctions requires additional late gene products (Merchlinsky, 1989).

Fenner *et al.* (1958) studied the effects of recombination between two strains of vaccinia virus. They found that haemagglutinin production and heat resistance were all-or-none characteristics. In these experiments, the reactions of mice and rabbits to infection with the recombinants were either typical of reaction to the less virulent parent or were of intermediate intensity. No correlation between the changes of virulence in mice and the changes of virulence in rabbits was found.

Since the experiments of Dumbell *et al.* (1964), there have been many more examples of recombination amongst a number of different species of orthopoxviruses in the laboratory. Bedson *et al.* (1964) described hybrids derived in vitro from the viruses of alastrim and rabbitpox and characterised the hybrids according to: pock type (size and ulceration), formation of pocks at 40^{o}C, haemagglutinin, thermal stability, plaque type, time of appearance of plaques, and virulence in rabbits and mice. The authors concluded that each of these markers was capable of segregating independently.

Chernos *et al.* (1985) characterised 19 recombinants obtained after coinfection of chicken embryo fibroblast cultures with vaccinia virus (neurovariant strain) and ectromelia virus. Four of these recombinants displayed pathogenicity in mice typical of ectromelia virus, while maintaining and even enhancing pathogenicity in rabbits typical of vaccinia virus ('double pathogenicity recombinants'). These four recombinants plus another four recombinants acquired a stable genetic trait which was not typical of the parental viruses: they were capable of inducing haemorrhagic lesions on the chorioallantoic membrane of chicken embryos. The DNA of the recombinants was shown to contain mostly vaccinia virus DNA with a single detected insert of ectromelia virus DNA. In the 'double pathogenicity' recombinants, this insert was located in the central part of the genome. In other experiments, cell cultures infected with ectromelia virus were transfected with rabbitpox virus DNA fragments. The result was the production of recombinants with a wide variety of properties. About 30 % of the recombinants had acquired ectromelia-specific pathogenicity (Chernos *et al.*, 1987).

As will be discussed, spontaneous recombination of poxviruses in animal hosts have been documented for capripox viruses and leporipoxviruses (malignant rabbit fibroma

virus). Moreover, one lethal case of infection with 'Lenny virus' was observed in Nigeria in 1969. This orthopox virus was characterised as a hybrid of smallpox and vaccinia viruses and presumably emerged from a double infection (Arita, 1982).

Strayer *et al.* (1983 a,b,c,d) isolated and investigated malignant rabbit fibroma virus, which is a recombinant between the two leporipoxviruses myxoma virus and Shope rabbit fibroma virus. When compared to its parents, the recombinant strain exhibited greater virulence with inherited pathogenic traits from both parental viruses. Malignant rabbit fibroma virus produces in infected rabbits a fibroma which progresses to a metastasising tumour in the invasive pattern of the myxoma virus. Dissemination of this malignant fibroma is associated with immunosuppression despite T-lymphocyte hyperplasia in the lymphoid tissues. The host range of malignant rabbit fibroma virus is similar to that of myxoma virus, which is broader than that of Shope fibroma virus. A supervening gram-negative infection with *Pasteurella multocida* eventually leads to death of the rabbit. In contrast to Shope fibroma virus, malignant rabbit fibroma virus is able to replicate to high titer in resting lymphocytes (Strayer *et al.*, 1985). The replacement of less than 5% of the myxoma genome with Shope fibroma virus terminal inverted repeat DNA sequences produced a dramatic change in the biology of the recombinant virus. Upton *et al.* (1986) sequenced these Shope fibroma virus terminal inverted repeats in the critical region. A subset of this region of the Shope fibroma virus genome was found to be closely related to an endogenous covalently closed circular plasmid species detected in uninfected rabbit cells. Upton *et al.* (1988) further cloned and sequenced all of the malignant rabbit fibroma virus recombination junctions, which are located near the left and right terminal inverted repeat regions, and presented a composite map with respect to the relevant gene products. One of these gene products was a protein with significant amino acid homology to the family of epidermal growth factors (EGF). The importance of such growth factors with respect to the proliferative phenotype of tumourigenic poxviruses remains to be clarified. Genes encoding EGF - like growth factors are not restricted to the tumourigenic poxviruses since vaccinia virus also encodes a polypeptide, designated vaccinia growth factor, which has significant amino acid homology to EGF and competes with it for binding to the EGF receptor. Opgenorth *et al.* (1992) showed that this growth factor is a major virulence factor in malignant rabbit fibroma virus infection.

Gershon *et al.* (1989) compared the maps of the genomes of four capripoxvirus isolates with individual genome types. Their comparison suggested that the progenitor of one of these isolates arose by genetic recombination between members of two of the other types.

Pastoret *et al.* (1995) claimed that the likelihood of an eventual recombination of the recombinant virus with a wild orthopoxvirus could be disregarded since there was no serological evidence of orthopoxvirus infection in the fox population. Boulanger *et al.* (1995) inoculated 4 foxes intradermally and 10 foxes orally with cowpox virus. Intradermally inoculated foxes developed only mild skin lesions with no evidence of virus replication. After oral inoculation, however, no lesions were observed although cowpox virus could be isolated from tonsils and from oropharyngeal swabs up to 5 days after inoculation, indicating that a transient local infection had occurred. All foxes seroconverted at 6 to 15 days after inoculation. It was concluded from these experiments that foxes were generally not very susceptible to cowpox virus and therefore at low risk for being host to an eventual recombination between cowpox

virus and VRG virus. These conclusions must, however, be re-examined in the light of the findings of Henning *et al.* (1995) in Germany who found antibodies against orthopoxvirus in 6.5% of 703 wild red fox (*Vulpes vulpes*) blood samples analysed. The antibodies found were later specified as anti-cowpox virus antibodies (see also Chapter II.2.3.1).

As bait uptake by various nontarget animals was frequently observed and as possible transmission to other species cannot be completely excluded, the risk of recombination in nontarget animals is a possibility. Rodents are known host reservoirs for different poxviruses; in Western Europe cowpox virus is maintained in wild rodents. Other animals preying on rodents also become potential sites for the recombination between vaccinia and cowpox viruses. It may be expected that vaccinia, cowpox and possible recombinant or chimeric viruses would share a common host range. But it is difficult to make any statements, as host range might not be a stable trait (Dales *et al.*, 1981).

The outcome from the recombination of two viruses cannot be predicted. It is even possible that progeny viruses with increased virulence compared to both parental viruses can result (for example: the spontaneous generation of malignant rabbit fibroma virus). There is experimental evidence on the generation of recombinant viruses with increased virulence. In one experiment, mice were either inoculated (footpad) simultaneously with two weakly neuroinvasive herpes simplex virus type 1 strains or with each strain alone (Javier *et al.* 1986). A total of 62% of mice having received a 1 : 1 mixture of the viruses died, whereas mice having received a similar or 100-fold higher dose of each individual strain alone survived. Of the 14 viruses isolated from the brains of 10 mice that had died, 11 were recombinants. Among the recombinants, 3 were lethal when reapplied to the footpads of mice. Henderson *et al.* (1990) also observed increased virulence in sheep after coinoculation of two avirulent vaccine strains of pseudorabies virus (alpha-herpesvirus). Other examples of recombination resulting in viruses with increased virulence compared to both parental strains were reviewed by Hahn (1992) and Kaplan (1989).

III. Safety assessment and probability considerations

III.1 Introduction

A thorough safety assessment on VRG use against rabies in wildlife involves identifying and estimating all possible hazards for humans and animals related to the release of this recombinant vaccine. This task can be subdivided into two important considerations:

1. The exposure potential or the likelihood of contact for humans and nontarget animals with VRG virus
2. The hazard potential for human and animal health, when exposure to virus takes place

An exposure scenario comprising all conceivable routes for spread and persistence of VRG in the biosphere is presented in figure 2. The key question asked in this scenario is whether or not the released VRG viruses will be able to spread and persist within the biosphere. Viral persistence would be the first step to other uncontrollable events such as contamination of non bait-ingesting animals through

the food chain or through contact; variations in the VRG phenotype and genotype; and an increased likelihood of recombination.

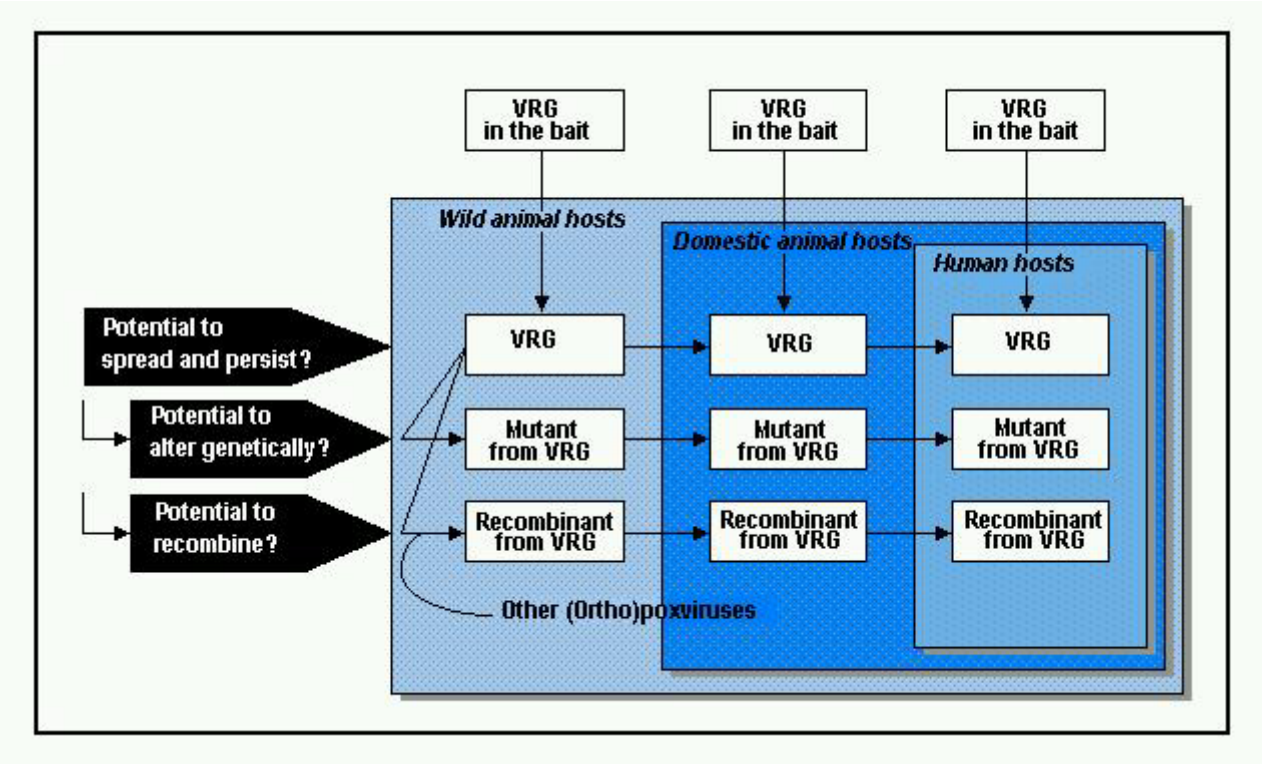

Fig. 2: Scenario and causal chain of possible routes for exposure to VRG and descendant viruses (mutant and recombinant) from VRG.

The likelihood for humans and animals to be exposed to VRG will depend on:

1. How well VRG replicates in the host
2. How long VRG persists in the biosphere

The hazard potential can be subdivided into danger from:

1. Direct contact with VRG
2. Mutants of the virus
3. Progeny virus derived from recombination

III.2 Exposure potential

III.2.1 Exposure potential to VRG virus or to progeny virus from VRG

III.2.1.1 Direct contact with VRG

VRG is a live virus vaccine and can replicate in the host. Because of its exceptional heat stability, which is the primary motivation for using Raboral® in Switzerland, stability and, hence, availability of the VRG vaccine virus under field conditions is high (several months).

Direct exposure of wild and domestic animals
VRG is taken up by target and nontarget animals directly from bait.

Direct exposure of humans
Humans are exposed to VRG through direct contact with the vaccine-containing bait which they find in the environment. Figures on the overall frequency of human contact with the baits are available from earlier rabies vaccination campaigns. Between 1992 and 1993, 4 vaccination campaigns against rabies were conducted in France. For one million air-distributed baits, a mean of 13.5 human contacts with bait was registered. The numbers taken from each campaign ranged from 28 contacts down to 3 contacts per million distributed baits and decreased from campaign to campaign; the downward trend was indicative of better public information programmes. Interestingly, more human contacts were reported with the attenuated rabies virus vaccines (23 contacts per million of distributed SAG 1 baits) than with the vaccinia based recombinant vaccine (8 contacts per million of distributed VRG baits). The difference in figures may be due to unreported cases of human contact with the VRG bait. As part of the information programme, the public was aware that treatment against rabies was not necessary after contact with the VRG bait. Furthermore, labels on the baits did not mention the word 'rabies' and, therefore, did not spur individuals to seek out attention from an authority.

Of the 63 baits (containing attenuated or recombinant rabies vaccine) found in France between 1992 and 1993, 26 baits were found by adults, 8 by children and 29 by dogs (Masson *et al.*, 1994). The baits had been retrieved mostly from gardens and school yards and other locations nearby human habitats (7 children; 14 adults). The 15 baits containing sachets with VRG vaccine were analysed: in 8 baits, the sachet was still intact, whereas in 7 baits, the sachets had been opened, enabling direct contact with the vaccine liquid. Dogs were more likely to tear open a vaccine sachet than humans. No clinical symptoms from vaccine exposure were observed in any of the persons having been in contact with the baits.

Vaccine baits in Switzerland will most likely be hand-distributed, thus allowing for a more targeted distribution scheme which would minimise the encounter of passers-by with the baits.

III.2.1.2 Replication and persistence potential

The fate of any released live virus is unpredictable. Whether or not the virus will be able to persist in the ecosystem or will vanish cannot be foreseen. It is known, however, that the vaccinia virus and, hence, VRG have not been indigenous in the European wildlife.

The vaccinia virus is a laboratory organism of unknown origin, yet it can replicate in an extremely wide range of animal hosts (mammals and birds). Assessments on the environmental and human health issues related to recombinant VRG release must evaluate the persistence of virus with its potential spread within target and nontarget organisms. As outlined below, transmission within target species is a rare event.

Subsequent indirect exposure of wild and domestic animals
With sufficient viral spread and persistence, VRG might enter the food chain or be transmitted from one animal to another. Domestic animals could be infected either by

bait uptake, by contact with infected wild animals or by feeding on infected animals (for example, cats on infected mice).

Experiments with VRG-infected animals, mostly foxes and raccoons, showed that virus could be isolated for a maximum of 2 days post-inoculation from tonsils and buccal mucosa, indicating that the time window for transmission was very short. Transmissibility of VRG seems to be low: one case of VRG transmission from fox to fox by biting was observed; occasional (rare) incidences of transfer from raccoon to raccoon (housed together, male - female pair, mother - offspring pair) have been reported. In several specific cases, it was possible to reisolate orthopox viruses several weeks after infection of susceptible animals. Virus latency is not a characteristic of vaccinia and VRG viruses, despite the tendency for DNA-viruses to be latent compared to RNA-viruses.

During the smallpox eradication campaign, some viral transmissions from vaccinated humans to animals such as cows, camels, rabbits, and buffaloes have occurred. In the case of Indian water buffaloes, the virus stabilised in the herd.

There seems to be a low, but non-excludable, likelihood for the persistence of VRG viruses in the biosphere.

Subsequent indirect exposure of humans
Provided there is sufficient viral spread and persistence, VRG virus might be transmitted to humans from domestic animals (infected by bait uptake, by preying on infected animals or by contact to wild animals) or after contact with infected wild animals (an extremely rare event).

From the interrupted experiment of VRG vaccination in cows on an experimental farm in Argentina, it was found that seroconversion but no health problems had occurred in at least one farm worker as well as in all contact animals (unpublished results).

One documented case with lethal outcome exists for cowpox virus transmission from a domestic cat to an immunosuppressed man, demonstrating the plausibility of this transmission route. Cats may be infected with orthopoxviruses from contact with rodents, which are known host reservoirs for these viruses. Assuming, however, a prevalence of orthopox viruses in rodents and considering that antibodies against orthopox viruses were found in 2 - 10 % of German cats, the incidence rate of human cowpox is presently very low.

The transmission of vaccinia virus from one human to another appears to be low; contact transmission of vaccinia virus from persons vaccinated against smallpox to susceptible contact persons has occurred but not often. This rate is 27 contact transmissions per million vaccinations, as established from smallpox vaccination campaigns.

III.2.1.3 Exposure potential to variants or recombinants from VRG virus

The DNA viruses generally have much greater genetic stability (the mutation rate being about 10^4 times lower) than RNA viruses. Within the (DNA) poxvirus family, however, considerable genetic variability can be seen. Spontaneous alterations of poxviruses, affecting cytopathology, virulence, structure and infectiousness, have

been reported. Selective pressure for viral gene mutation arise in part from environmental (host) conditions under which viruses proliferate. Vaccinia virus has a relatively high variability in the terminal region of the genome. Vaccinia virus variants (see chapter II.1.1) have been reported which differ in host range (depending on host-passages), tissue tropism (due to differences in maintenance), temperature sensitivity and drug resistance. Not all alterations in virus characteristics result from changes in the genotype; some may come from non-genetic material from the host cell incorporated by the vaccinia virus during replication. The introduced rabies glycoprotein G characteristics in VRG viruses have been tested and found to be stable.

Members of the poxvirus family are known to recombine easily. New hybrid viruses created through recombination could potentially spread undetected through wildlife or could be transmitted to domestic animals. Rare examples of recombination between different poxviruses in animal hosts have been documented, although the probability of two viruses infecting the same cell at the same time (pre-requisite for recombination) under natural conditions remains very low. Recombination of VRG with viruses belonging to other genera than orthopoxviruses is not likely.

Still, recombination cannot be excluded, and the list of likely vaccinia virus recombination partners must be analysed. Distribution of orthopoxviruses, the most favourable canditates, varies according to different geographical and ecological conditions.

The cowpox virus has a wide host range; cows and humans are only sporadic indicator hosts, whereas rodents serve as natural reservoirs. The rare disease caused by cowpox virus is geographically limited to Britain and Western Europe. A total of 16 cases of human infection have been reported between 1969 and 1981. In 13 of the cases, the source of infection remains unknown. Although human cowpox is generally acquired from infected cattle, the disease sometimes occurs without any contact with diseased cows. Surveys in Germany showed seropositivity against cowpox virus in 6.5 % of the foxes and in 9.9 % of the cats tested. Cowpox may also be maintained in small mammals without causing disease in them.

Another orthopoxvirus found in Europe is ectromelia virus, which is not pathogenic for humans but is endemic in mice. Orthopoxviruses not known to be endemic in European wild life are: buffalopox (presumably vaccinia) virus; California vole pox virus; camelpox virus; rabbitpox ('neurovaccinia') virus (in European laboratories); raccoon pox virus; tatera pox virus; Uasin Gishu pox virus; vole pox virus; and monkeypox virus.

If it is possible to extrapolate from observations regarding other orthopoxviruses, that small mammals and especially rodents could also be potential host reservoirs for VRG or its emerging recombinant progeny. This population of small mammals could be a starting point for indirect viral spread through either contact transmission or the food chain (chapter III.2.1.2).

III.2.2 Exposure potential to attenuated rabies virus

The SAD strain of rabies virus and its derivatives have been used as vaccines in the field since 1978, the year of the world-wide first field trial for oral rabies immunisation of foxes in Switzerland.

When the conventional rabies vaccines based on attenuated rabies virus were first introduced, there was some concern about target and nontarget animals (mammals) contracting the disease from the vaccine virus. There was also some speculation about bait-ingesting rodents becoming a rabies reservoir. Because of the uncontrolled rabies epizootic at that time, the attenuated rabies vaccine was introduced despite awareness of the potential hazards. During more than 10 years of carefully monitored field application of SAD Berne in Switzerland, it was possible to isolate this strain from three affected animals (one domestic cat, one stone marten and one fox).

The likelihood of human exposure to SAD vaccine virus by direct bait contact has been described earlier (III.2.1.1). As no persistence of the attenuated rabies viruses in the environment has been observed, the probability of human exposure to this vaccine virus remains low. The animals infected with wild-type rabies virus most likely to come into contact with humans are, in order: domestic animals, mostly cats (European dogs being generally vaccinated and better supervised); grazing livestock; wild animals (seldom). The risk of rabies transmission from a rodent is very low.

RNA viruses, such as rabies virus, exhibit much higher genetic variability than DNA viruses, such as vaccinia virus. One reason for the higher mutation rate seen in RNA viruses is the lack of proof-reading activity during replication. High genetic variability arises due to errors made in viral genome copies. Extensive in vitro and in vivo passages of the attenuated rabies viruses is another source of selective pressure on the genome. Severely altered cell and tissue tropism have been observed after multiple passages; fortunately, these mutants have not been able to persist so far in wildlife. An attenuated rabies vaccine carries with it a greater risk for reversion to virulence from random mutations. This problem has been obviated by the use, in Switzerland, of a new strain, SAG 2, which has a lower potential for reversion to virulence.

Recombination is more likely with poxviruses than with rhabdoviruses. In unsegmented negative-strand RNA viruses such as rhabdoviruses recombination has never led to a new virus capable of replication. Defective interfering particles created by the recombination process, however, have been observed with high frequency (Conzelmann *et al.*, 1994). Yet, recombination cannot be completely ruled out.

III.3 Hazard potential

Once exposure has taken place, a possible hazard might materialise. In this second part of the safety assessment, a review of hazards related to VRG exposure must be performed with attempt to answer the following questions:

1. What are all possible hazards to human and animal health (impact aspects)?
2. What is the likelihood of each type of hazard?

All statements given on the impact aspects are based on knowledge drawn from the extensive literature review presented in part II of this report where more detailed information and references can be found.

As outlined in the exposure scenario, the hazard potential can be subdivided into possible hazards arising from: VRG virus, progeny viruses derived from mutation of VRG in the field, or progeny viruses derived from recombination of VRG in the field.

III.3.1 Hazard potential of VRG virus

III.3.1.1 Possible hazards from the VRG vaccine virus

Possible impacts on wildlife
The vaccination of a wide range of animal species with VRG was proven to be innocuous in a controlled setting. As the ecology and adaptation behaviour are complex in different wild animal species, it is not possible to predict the long-term biological outcome of systematic vaccinations with VRG. Moreover, it is impossible to speak of absolute safety with regard to any biological systems.

Vaccinia virus (strains Wistar and Copenhagen) has been shown to decrease the fertility of mice through viral replication in the ovary and testes. Another observed hazard impact is the ability of the same virus to induce varying degrees of species-specific pathogenicity. An example of this is the earlier described outbreak of an orthopox virus infection (presumed cowpox) in the Moskow Zoo.

Possible impact on humans
Vaccinia virus is a class 2 risk group organism. Microorganisms of this class do not pose any hazard to healthy individuals, but may affect certain predisposed individuals. It is assumed that the consequences following exposure to VRG in humans will vary from case to case, with symptoms ranging from almost none to severe disease.

During the vaccination programme against smallpox, inoculation of persons with vaccinia virus usually took place through intradermal application. There were some incidences of **inadvertent auto- and heteroinoculation** involving mostly the face: eyelid, nose, mouth; genitalia; and rectum. When vaccinia virus induced keratitis or scarring of the cornea, impaired vision or even loss of the eye occurred. Data from a survey using the Wyeth strain showed that over 60% of contact transmission resulted in uncomplicated inadvertent inoculation, whereas approximately 30% resulted in eczema vaccinatum, which sometimes was even more severe among contacts than among vaccinated persons.

Postvaccinial encephalitis has been recognised as a serious neurological complication arising from immunisation using the vaccinia virus, yet it has been impossible to characterise the individuals at risk for this disorder. Based on the statistics from vaccination programmes involving mainly children, the figures for postvaccinial encephalitis lie between 3 to 60 cases per million vaccinees. Case fatality rate in patients aged less than 2 years was more than 50%; surviving patients sustained handicaps upon recovery. In patients aged over 2 years the case fatality rate was about 35% with generally complete recovery of the surviving patients. Adult persons receiving first-time vaccinia vaccinations seem to be more susceptible to this

complication than children. It is recommended that first-time adult vaccinees receive pretreatment with inactivated vaccinia antigen or be given a simultaneous vaccinia immunoglobulin treatment when vaccination is necessary.

More neurological side effects have been associated with the Copenhagen strain of vaccinia than other vaccinia viruses. This was the reason why the Dutch authorities in 1963 replaced the Copenhagen strain by the less pathogenic Elstree strain for smallpox vaccinations.

Eczema vaccinatum occurred among persons with (a history of) atopic eczema either after vaccination or after contact with vaccinated persons. For eczema vaccinatum, incidence rates between 10 and 120 cases per million vaccinations have been given. Case fatality seemed to vary from one study to another. Figures from 5 to 40 % with reduction to 7% after treatment with vaccinia immunoglobulin have been reported. The outcome of eczema vaccinatum may actually be more severe for contacts than for vaccinated persons. Infants with (a history of) atopic eczema form a high risk group.

Progressive vaccinia occurred almost exclusively among immunodeficient persons. The incidence rate observed, about 1 case per million, was low, but the case fatality rate approached 90%.

Generalised vaccinia occurred in patients with underlying illnesses and was generally selflimiting.

Members of a designated **risk group** are more likely to suffer serious consequences resulting from viral transmission (for example, the lethal cowpox virus infection of an immunosuppressed young man by a cat). Immunodeficient persons, infants with (a history of) atopic eczema, and pregnant women form the three major risk groups.

Few persons are expected to come into contact with the VRG virus compared to the tremendous number of persons inoculated with vaccinia virus during the smallpox eradication campaign. Extensive data are available on adverse reactions to the smallpox vaccination, yet it is not possible to forecast the nature and extent of health complications associated with exposure to VRG. Significant differences exist between the intention and use of the vaccine strains for smallpox and rabies.

1. The application mode:
   During the smallpox eradication programme, vaccinees were inoculated with vaccinia virus by subcutaneous application. During contact with VRG, humans might be infected through lesions in the skin. Other routes of infection might be the same ones as those observed in cases of inadvertent auto- or heteroinoculation: face (eyelid, eyes, nose, and mouth); genitalia; and rectum.
2. The target group:
   Because the smallpox vaccination campaigns have come to an end, and individuals are no longer systematically immunised, there is an increasing number of adult persons who are vaccinia-naive. As discussed earlier, adults having no established vaccinia antibody titers (no previous vaccination) are at greater risk for developing neurological complications, such as postvaccinial encephalitis, when exposed to vaccinia for the first time.
   Whereas it was possible during the smallpox vaccination campaign, to avoid

vaccinating individuals belonging to a risk group, the same controlled selection is not possible for exposure to VRG. Individuals, albeit a low number, come in contact with VRG on a random basis. Members of a risk group might find the baits as well or might have contact with VRG-infected domestic animals. To be taken into consideration is the significant increase in the last years of persons HIV-positive or with AIDS. This, along with a slight increase in cases of atopic eczema, results in a higher proportion of persons belonging to risk groups.

3. The virus used in the vaccine:
   VRG is expected to be less pathogenic than wild-type vaccinia strain Copenhagen. In the VRG genome, the thymidine kinase gene of vaccinia is insertionally inactivated. Observations from animal models suggest, that tk^- vaccinia virus is less pathogenic than wild-type vaccinia virus.
   The genetic sequence inserted into vaccinia codes for the rabies glycoprotein G. It is generally assumed that glycoprotein G is produced in the host cell but not incorporated into the envelope of the recombinant virus. The presence of glycoprotein G on wild-type viral envelope is suspected to affect tissue tropism and to cause neurovirulence. Until now, VRG has never been detected in the brain and central nervous system of the animals tested after vaccination.

The exposure of humans and animals to VRG (vaccinia) could influence the efficacy of future vaccinations using other recombinant vaccinia viruses (chapter II.4.5). Vaccination of vaccinia-naive individuals with vaccinia-based vaccines is expected to be more efficacious than vaccination of vaccinia-primed individuals.

III.3.1.2 Possible hazards from genetic (and phenotypic) variation

Genetic stability of the glycoprotein G gene introduced into VRG virus vaccine has been tested to be stable.

Spontaneous alterations of poxviruses in cytopathology, virulence, structure and infectiousness have been reported. For vaccinia virus (see chapter II.1.1), variations have been observed in host range (according to host-passages), tissue tropism (according to differences in maintenance), temperature sensitivity and drug resistance. Individual strains of vaccinia virus may also differ in pathogenicity.

III.3.1.3 Possible hazards arising from recombination

Recombination of VRG with another (ortho-)poxvirus might take place on rare occasions. The probability and possible outcome of recombination will depend on the regional virus pools available for recombination.

In the European wildlife virus pool, possible partners for VRG would be cowpox virus or ectromelia virus. Cowpox is a rare disease found only in Britain and western Europe; human cowpox usually results in localised skin lesions sometimes accompanied by fever and other symptoms. This disease affects children more severely than adults. Ectromelia virus is found in mice and is not pathogenic to humans.

In 1991, a lethal case of transmission of a virus, similar to cowpox, from a cat to an immunosuppressed man was reported. Both cows and humans are only sporadic indicator hosts of the cowpox virus, whereas rodents are known stable natural

reservoirs. Cowpox may also be maintained in small wild animals without causing disease in them. The host range of the cowpox virus is wide; in the domestic cat , the cowpox (or catpox) virus causes minor symptoms, in large felines (zoo), the virus causes a fatal fulminating pulmonary infection.

Monkeypox virus has become the most dangerous orthopoxvirus since eradication of the variola virus; it is indigenous in tropical rain forest areas of West and Central Africa. Releasing VRG in tropical rain forest areas would therefore pose a greater hazard potential than releasing VRG in Europe. Human infection with monkeypox virus is clinically indistinguishable from smallpox. Fortunately, this zoonosis remains rare with even rarer incidences of human-to-human transmission. In Europe, only captive animals have been shown to be sources of monkeypox virus.

As the example of malignant rabbit fibroma virus illustrates (chapter II.5.1), it is impossible to predict the outcome of recombination events. Progeny viruses might even have increased virulence when compared to both parental viruses. During recombination events, alterations in viral transmissibility or pathogenicity define the hazard potential.

III.3.2 Possible hazards arising from attenuated rabies virus vaccine

The presently used live but attenuated rabies virus vaccines retain the potential for inducing fatal rabies disease. In animal experiments with rodents, residual pathogenicity has been demonstrated for both SAD strains (SAD Berne and SAD 19), whereas SAG 1 and VRG were shown to be innocuous.

The attenuated vaccine strains may also still be pathogenic to humans, and persons exposed to SAD derived attenuated strains of rabies virus must be treated with a conventional inactivated rabies vaccine. There has been at least one case of severe infection of a technician after he was exposed to an aerosol of modified live rabies virus strains, derived from SAD commercial veterinary rabies strain. The infection developed in spite of earlier multiple preventive vaccinations (Tillotson *et al.*, 1977). Persons forming a risk group for developing adverse health effects from exposure to the attenuated rabies vaccine virus are the same as those described for vaccinia virus.

As mentioned earlier, RNA viruses are less genetically stable than DNA viruses. SAG 2 remains presently the most stable attenuated RNA rabies virus vaccine strain used with respect to reversion to virulence.

Although the recombination of two unsegmented negative-strand RNA viruses, such as rhabdoviruses, has never been observed to yield a new virus capable of replicating, one should be aware that recombination might take place. The potential hazard would be fatal rabies; the most dangerous virus trait which could be altered would be transmissibility.

III.3.3. Possible consequences common to attenuated and recombinant vaccines against rabies

Both attenuated and recombinant vaccines induce either an early or a late death phenomenon in asymptomatic animals which are already incubating rabies; no emergence of asymptomatic carriers is therefore expected.

The eradication of rabies may have direct consequences on the ecological balance; lack of disease favours an increase in the fox population. This increase might further induce other ecological changes such as, for example, an increased spread of fox tapeworm.

III.4 Risk appraisal

All possible vaccination strategies have to be included in a risk appraisal. For rabies there are three existing alternatives:

1. Oral vaccination of wildlife against rabies using live, attenuated rabies virus vaccine
2. Oral vaccination of wildlife against rabies by live, recombinant VRG vaccine
3. No vaccination of wildlife against rabies

Figure 3 schematically presents the procedure followed throughout this report; the impact aspect of VRG vaccine is the most intensively covered subject. Potential hazards arising from VRG release and use are briefly compared to potential hazards from vaccination with attenuated rabies vaccines or no vaccination at all. The assessment is qualitative due to the complexity of the problem and to the absence of appropriate data. Moreover, some of the fundamental questions discussed are subject to a broad interpretation range.

The information on VRG presented in this part of the report was introduced in chapter I.2.2, then reviewed extensively in part II and summarised in chapters III.2 and III.3. For more detailed information and greater understanding of the various impact aspects concernig VRG discussed here, the reader is referred to the literature review of part II. Because not all information required can be obtained by direct experience with VRG virus, this report draws upon knowledge of the vaccinia virus (parental organism for VRG); (ortho)poxviruses (more general) and the rabies glycoprotein G (insert).

The information concerning the attenuated rabies vaccine was given in chapters I.2.1, III.2 and III.3.

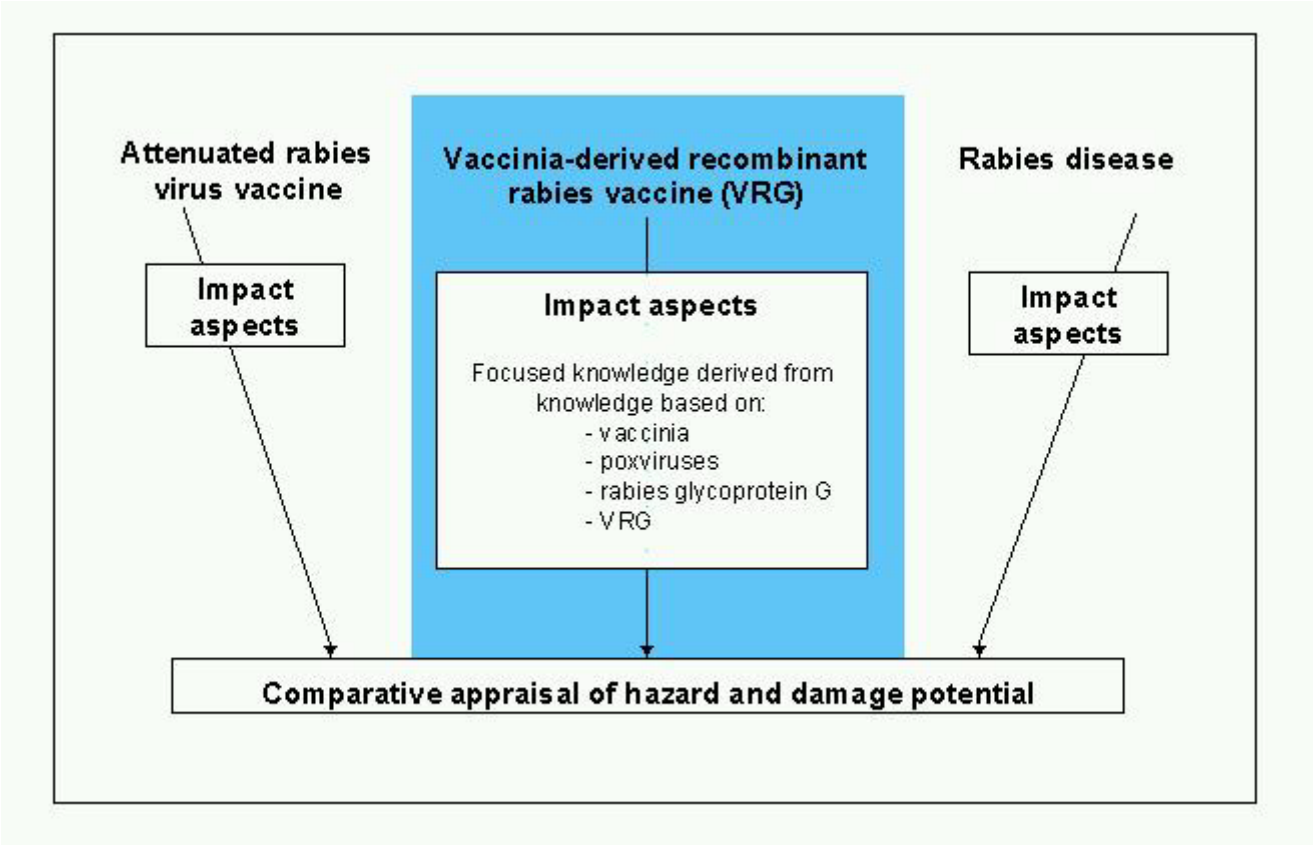

Fig. 3: Procedure of safety assessment.

As outlined above, both attenuated and recombinant (VRG) live vaccines against rabies have their associated advantages and disadvantages. The bulk of qualitative assessment for VRG is based on the accumulated data from the smallpox vaccination campaigns, for which the risk and outcome of vaccine-related complications are available. Another source of data are the documented cases related to the contact of humans with vaccine-containing bait in the environment. No quantitative data can be given regarding the probabilitiy for vaccine-induced complications and their extent. Some important characteristics of live attenuated and recombinant vaccines are summarised in Table 4.

Table 4: *A synopsis: Key features of oral attenuated rabies vaccine and oral vaccinia-based recombinant rabies vaccines.*

	VRG and (*) vaccinia respectively	**Attenuated rabies vaccine**
Year of first release:	1987	1978
Origin	* Laboratory organism of unknown origin; belongs to the genus of orthopoxviruses	Derived from the SAD strain of wild-type rabies virus by extensive in vivo and in vitro passages
Exposure potential:		

Durability under field conditions	High (first significant decrease of virus titer after 4 months) High thermostability	Low Low thermostability
Direct bait uptake by nontarget animals	Yes	Yes
Persistence in the biosphere	Not yet observed, but cannot be excluded	Not yet observed, but cannot be excluded
Host range	Mammals and birds	Mammals
Transmissibility animals	Low	Low
Transmissibility humans	* Very low	very low
Genetic stability	High (DNA virus)	Low (RNA virus)
Recombination potential	No recombination in the field observed yet. With poxviruses under laboratory conditions recombination occurs frequently. Recombination with other (ortho)poxviruses present in wildlife is possible although with low incidence (infection of same host cell at same time).	No recombination in the field observed yet. Defective particles frequently observed from recombination, but recombination has never yielded a new rhabdovirus which was able to replicate
Possible outcome of recombination	Unpredictable and expected to vary according to geographical locations (dependent on availability of indigenous viruses as partners for recombination)	Unpredictable
Possible hazards in animals	Innocuous to a wide range of animals tested * The fertility of infected small rodents may be impaired	All vaccine strains are attenuated but retain the potential for inducing fatal rabies. Depending on the vaccine strain there was residual pathogenicity in rodents.
Possible hazards in humans	* Individual reactions range from minor (most cases) to lethal (rare cases) (details see part II). * Possible complications: encephalitis, progressive vaccinia, eczema vaccinatum, generalised vaccinia, and localised adverse effects (e.g.	All vaccine strains are attenuated but retain the potential for inducing fatal rabies. If a wild-type rabies virus infection is not treated before the virus reaches the nervous system, outcome is almost invariably fatal

	scarring of the cornea) VRG is attenuated (tk-locus) with respect to its parental organism, vaccinia strain Copenhagen	
Therapy	* Immunoglobulin (if available) or Marboran * Not always effective	Active and passive immunisation are highly effective if administered before the virus reaches the nervous system
Risk groups	* Immunodeficient persons, infants with (a history of) atopic eczema, pregnant women * For postvaccinial encephalitis no risk group has been identified, but adults are more susceptible	Basically the same risk groups as for VRG

In light of the extensive information presented in this report it is clear that the risks and benefits relating to VRG release need to be carefully examined. Although a comprehensive risk-benefit appraisal has not been the objective of this report, substantial information has been gathered and presented here which would be useful in discussions concerning impacts from the environmental release of VRG in Switzerland.

It is unrealistic and impossible to ensure the absolute safety of vaccinations, since some health complications are known to be caused by vaccines. The risk-benefit analysis must remain focused on the greater potential health threat. For the smallpox vaccination campaigns, the risk of vaccine-related complications had to be weighed against the risk of a smallpox epidemic. Similarly, the risk of exposure to VRG has to be weighed against the risk of infection with rabies virus.

The European Community (except Germany) and North American authorities have decided in favour of the introduction of recombinant vaccinia-rabies glycoprotein G vaccine. Support for this decision came in part from a study conducted by the APHIS (Animal and Plant Health Inspection Service); the consensus was that introduction of the recombinant vaccine would have 'no significant impact'. VRG is presently used for the oral vaccination of wild animals against rabies in France, Belgium and in the U.S.A.

Rabies remains a dangerous disease for all mammals, including humans. Uncontrolled wildlife rabies has a significant impact on public health (Smith *et al.*, 1995). When transmitted to man the rabies virus becomes invariably fatal when it reaches the nervous system. Because the incubation time for rabies in man is long, passive and active immunisation remain highly efficient therapeutic means for preventing the disease in exposed humans. Still, postexposure treatment of humans is unpleasant and costly.

From the cost-benefit point of view, the vaccination of foxes can be shown to be cost-efficient: uncontrolled rabies is responsible for important livestock loss; vaccinating livestock is comparatively more expensive; and post-exposure treatments for affected

persons are costly (Zanoni, 1996; Uhaa *et al.*, 1992; Dufour *et al.*, 1989; Aubert *et al.*, 1993).

Owing to the success of previous intensive vaccination campaigns, the incidence of rabies cases in Switzerland has decreased considerably. The risk of a new rabies epizootic is, however, ever present. Solitary cases of rabies in a population of foxes with an increasing number of unimmunised foxes might again cause spread of the disease; this new rabies epizootic might even be worse because, due to the success of rabies vaccination, the population density of foxes has been increasing considerably. When and if vaccination against rabies should be terminated after the last case registered in the wildlife requires careful assessment according to rabies epidemiology.

The choice of the vaccinia virus strain Copenhagen for the construction of the recombinant vaccine may not seem ideal. Not only has the Copenhagen strain of vaccinia been associated with a relatively higher incidence rate of neurological symptoms compared to other vaccinia strains, but vaccinia virus itself has a broad host range. These criticisms are valid, but VRG is a first generation vaccine that has successfully passed stringent safety testing. Research is ongoing for other recombinant vaccines based on highly attenuated vaccinia virus strains, such as the non-replicating strain MVA, derived from vaccinia virus strain Ankara, which also efficiently expresses recombinant genes. Due to the high amount of safety testing needed for the registration of a new vaccine, the replacement of VRG with other recombinant vaccines will take some time. Further research on the development of efficient **inactivated** vaccines for oral vaccination is required and encouraged by WHO (WHO, 1993b).

Immunoglobulin therapy has been shown to be efficient for certain cases of complications related to smallpox vaccination using vaccinia virus. The case fatality rate of eczema vaccinatum was notably diminished using this therapy. In the event that the VRG virus is released, a stock of available immunoglobulin for human treatment would be highly recommended.

IV. References

Anderson, R.M. Immunization in the field. Nature 354: 502-503; 1991.

Andrew, M.E.; Coupar, B.E.H.; Boyle, D.B. Immunogenicity and Antigen Presentation . In: Binns, M.M.; Smith, G.L., eds. Recombinant Poxviruses. Boca Raton: CRC Press; 1992: p. 207-234.

Angulo, J.J.; Pimenta-de-Campos, E.; deSalles-Gomes, L.F. Postvaccinial Meningo-Encephalitis. JAMA 187: 151-153; 1964.

Arita, I.; Gromyko, A. Surveillance of orthopox virus infections, and associated research, in the period after smallpox eradication. Bulletin WHO 60: 367-375; 1982.

Artois, M.; Aubert, M.F.A.; Poulle, M.L. Essais de dépose d'appâts vaccinaux contre la rage contenant le vaccin recombinant vaccine-rage (Juillet 1988-Juillet 1989). Centre National d'Etudes Vétérinaires et Alimentaires; Laboratoire d'Etudes sur la Rage et la Pathologie des Animaux Sauvages: Rapport final. 1990.

Artois, M.; Guittré, C.; Thomas, I.; Leblois, H.; Brochier, B.; Barrat, J. Potential pathogenicity for rodents of vaccines intended for oral vaccination against rabies: a comparision. Vaccine 10: 524-528; 1992.

Aubert, M. Towards Elimination of Wildlife Rabies in France by the Use of Oral Vaccination with Vaccinia Recombinant Rabies and Highly Modified Rabies Strain Vaccines. Presented at: 55. Jahresversammlung der Schweizerischen Gesellschaft für Mikrobiologie, Bern. 1. März 1996.

Aubert, M.F.A.; Masson, E.; Artois, M.; Barrat, J. Oral Wildlife Rabies Vaccination Field Trials in Europe, with Recent Emphasis on France. In: Rupprecht, C.E.; Dietzschold, B.; Koprowski, H., eds. Lyssaviruses. Springer-Verlag Berlin Heidelberg: Current Topics in Microbiology and Immunology; 1994: p. 219-243.

Aubert, M.; Masson, E.; Vuillaume, Ph.; Artois, M.; Barrat, J. Les acquis de la prophylaxie contre la rage vulpine en France. Méd Mal Infect. 23; Spécial: 537-545; 1993.

BAG, anon. Empfehlungen für das Vorgehen nach Exposition mit einem Tollwut-Lebendimpfstoff (der nur in der Fuchs-Impfkampagne verwendet wird). Bulletin des Bundesamtes für Gesundheitswesen 34: 621-625; 1993.

Ball, L.A. High-Frequency Homologuous Recombination in Vaccinia Virus DNA. J. Virol. 61: 1788-1795; 1987.

Baxby, D. Poxvirus Hosts and Reservoirs. Brief Review. Archives of Virology 55: 169-179; 1977.

Baxby, D. Safety of recombinant vaccinia vaccines. Lancet 337: 913; 1991.

Baxby, D.; Gaskell, R.M.; Gaskell, C.J.; Bennett, M. Ecology of Orthopoxviruses and Use of Recombinant Vaccinia Vaccines. Lancet October 11: 850-851; 1986.

Baxby, D.; Hill, B.J. Characteristics of a New Poxvirus Isolated from Indian Buffaloes. Archiv für die gesamte Virusforschung 35: 70-79; 1971.

Bedson, H.S.; Dumbell, K.R. Hybrids Derived from the Viruses of Alastrim and Rabbit Pox. Journal of Hygiene (Cambridge) 62: 141-146; 1964.

Behbehani, A.M. The Smallpox Story: Life and Death of an Old Disease. Microbiological Reviews 47: 455-509; 1983.

Bennett, M.; Gaskell, C.J.; Gaskell, R.M.; Baxby, D.; Gruffydd-Jones, T.J. Poxvirus Infection in the Domestic Cat: Some Clinical and Epidemiological Observations. The Veterinary Record 118: 387-390; 1986.

Bergoin, M.; Dales, S. Comparative Observations on Poxviruses of Invertebrates and Vertebrates. In: Maramorosch, K.; Kurstak, E., eds. Comparative Virology. New York: Academic Press; 1971: p. 169-205.

Berufsgenossenschaft der chemischen Industrie. Sichere Biotechnologie. Eingruppierung biologischer Agenzien: Viren. Merkblatt B 004 (4/91); 1991.

Binns, M.M.; Smith, G.L. eds. Recombinant Poxviruses. Boca Raton: CRC Press; 1992.

Blancou, J.; Kieny, M.P.; Lathe, R.; Lecocq, J.P.; Pastoret, P.P.; Soulebot, J.P.; Desmettre, P. Oral vaccination of the fox against rabies using a live recombinant vaccinia virus. Nature 322: 373-375; 1986.

Blaskovic, D.; Bock, H.E.; Fanconi, G.; Friolet, B.; Germer, W.D.; Gibbels, E.; Gsell, O.; Henneberg, G.; Knüttgen, H.; Libikova, H.; Malamos, B.; Mayer, J.B.; Mohr, W.; Oehme, J.; Rossi, E.; Scheid, W.; Seifert, G.; Siegert, R.; Stüttgen, G.; Tönz, O.; Weisse, K. Krankheiten durch Viren, Band I. Teil 1: Krankheiten durch nachgewiesene Viren. Berlin, Heidelberg, New York: Springer-Verlag; 1967: p. 697 ff.

Boulanger, D.; Brochier, B.; Crouch, A.; Bennett, M.; Gaskell, R.M.; Baxby, D.; Pastoret, P.-P. Comparision of the susceptibility of the red fox (*Vulpes vulpes*) to a vaccinia-rabies recombinant virus and to cowpox virus. Vaccine 13: 215-219; 1995.

Breitenmoser, U.; Kappeler, A.; Müller, U.; Zanoni, R. Tollwut und ihre Bekämpfung in der Schweiz. Wildbiologie in der Schweiz 6/26: 1-14; 1996.

Brochier, B.; Kieny, M.P.; Costy, F.; Coppens, P.; Bauduin, B.; Lecocq, J.P.; Languet, B.; Chappuis, G.; Desmettre, P.; Afiademanyo, K.; Libois, R.; Pastoret, P.-P. Large-Scale Eradication of Rabies Using Recombinant Vaccinia-Rabies Vaccine. Nature 354: 520-522; 1991.

Brochier, B.; Thomas, I.; Bauduin, B.; Leveau, T.; Pastoret, P.-P.; Languet, B.; Chappuis, G.; Desmettre, P.; Blancou, J.; Artois, M. Use of a vaccinia-rabies recombinant virus for the oral vaccination of foxes against rabies. Vaccine 8: 101-104; 1990.

Brochier, B.; Blancou, J.; Thomas, I.; Languet, B.; Artois, M.; Kieny, M.-P.; Lecocq, J.-P.; Costy, F.; Desmettre, P.; Chappuis, G.; Pastoret, P.-P. Use of Recombinant Vaccinia-Rabies Glycoprotein Virus for Oral Vaccination of Wildlife against Rabies: Innocuity to Several Non-Target Bait Consuming Species. Journal of Wildlife Diseases 25: 540-547; 1989a.

Brochier, B.M.; Blancou, J.; Aubert, M.F.A.; Kieny, M.P.; Desmettre, P.; Pastoret, P.-P. Interaction between Rabies Infection and Oral Administration of Vaccinia-Rabies Recombinant Virus to Foxes (*Vulpes vulpes*). Journal of General Virology 70: 1601-1604; 1989b.

Brochier, B.; Thomas, I.; Iokem, A.; Ginter, A.; Kalpers, J.; Paquot, A.; Costy, F.; Pastoret, P.-P. A Field Trial in Belgium to Control Fox Rabies by Oral Immunisation. The Veterinary Record 123: 618-621; 1988a.

Brochier, B.M.; Languet, B.; Blancou, J.; Kieny, M.P.; Lecocq, J.P.; Costy, F.; Desmettre, P.; Pastoret, P.-P. Use of Recombinant Vaccinia-Rabies Virus for Oral

Vaccination of Fox Cubs (*Vulpes vulpes*) against Rabies. Veterinary Microbiology 18: 103-108; 1988b.

Brown, F.; Schild, G.C.; Ada, G.L. Recombinant vaccinia viruses as vaccines. Nature (London) 319: 549-550; 1986.

Bruggemann, E.P. Environmental Safety Issues for Genetically Modified Animals. J. Anim. Sci. 71(Suppl. 3): 47-50; 1993.

Buller, R.M.L.; Palumbo, G.J. Safety and Attenuation of Vaccinia Virus. In: Binns, M.M.; Smith, G.L., eds. Recombinant Poxviruses. Boca Raton: CRC Press; 1992: p. 235-267.

Buller, R.M.L.; Palumbo, G.J. Poxvirus Pathogenesis. Microbiological Reviews 55: 80-122; 1991.

Buller, R.M.L.; Moss, B. Genetic Basis for Vaccinia Virus Virulence. In: Quinnan, G.V. ed. Vaccinia Viruses as Vectors for Vaccine Antigens. Nov. 13-14 1984: Proceedings of the Workshop on Vaccinia Viruses as Vectors for Vaccine Antigens, Chevy Chase. New York: Elsevier; 1985a: 37-46.

Buller, R.M.L.; Smith, G.L.; Cremer, K.; Notkins, A.L.; Moss, B. Decreased virulence of recombinant vaccinia virus expression vectors is associated with a thymidine kinase-negative phenotype. Nature 317: 813-815; 1985b

Centers for Disease Control, Atlanta. Vaccinia (Smallpox) Vaccine. Recommendations of the Immunization Practices Advisory Committee (ACIP). MMWR (Morbidity and Mortality Weekly Report) 40 (RR-14): 1-10; 1991.

Chernos, V.I.; Antonova, T.P.; Senkevich, T.G. Recombinants between Vaccinia and Ectromelia Viruses Bearing the Specific Pathogenicity Markers of Both Parents. J. Gen. Virol. 66: 621-626; 1985.

Chernos, V.I.; Senkevich, T.G.; Chelyapov, N.V.; Antonova, T.P.; Vovk, T.S.; Mitina, I.V. Biologic Properties and Genome Structure of the Recombinants between Ectromelia and Rabbitpox Viruses. Acta Virologica 31: 193-202; 1987.

Collier, L.H. Safety of recombinant vaccinia vaccines. Lancet 337: 1035-1036; 1991.

Conzelmann, K.K.; Schnell, M.; Mebatsion, T.; Cox, J. eds. Genetische Stabilität des Tollwutvirus. In: Biologische Sicherheit, Band 3. Forschung Biotechnologie. Tübingen: Bundesforschungsanstalt für Viruskrankheiten der Tiere; 1994.

Cooney, E.L.; Collier, A.C.; Greenberg, P.D.; Coombs, R.W.; Zarling, J.; Arditti, D.E.; Hoffman, M.C.; Hu, S.-L.; Corey, L. Safety of and immunological response to a recombinant vaccinia virus vaccine expressing HIV envelope glycoprotein. Lancet 337: 567-572; 1991.

Coulon, P.; Rollin, P.E.; Flamand, A. Molecular Basis of Rabies Virus Virulence. II. Identification of a Site on the CVS Glycoprotein Associated with Virulence. Journal of General Virology 64: 693-696; 1983.

Crawford, M. Overseas Field Tests under Fire. Science 234: 1068-1069; 1986.

Czerny, C.-P. Risikobeurteilung des rekombinanten Tollwutimpfstoffes "Raboral". Schriftliche Stellungnahme zuhanden des Schweizerischen Bundesamtes für Umwelt, Wald und Landschaft. 14 S.; 1996.

Czerny, C.-P.; Eis-Hübinger, A.M.; Mayr, A.; Schneweis, K.E.; Pfeiff, B. Animal Poxviruses Transmitted from Cat to Man: Current Event with Lethal End. J. Vet. Med. B 83: 421-431; 1991.

Czerny, C.-P.; Johann, S.; Hölzle, L.; Meyer, H. Epitope Detection in the Envelope of Intracellular Naked Orthopox Viruses and Identification of Encoding Genes. Virology 200: 764-777; 1994.

Dales, S.; Pogo, B.G.T. Biology of Poxviruses. In: Kingsbury, D.W.; Zur Hausen, H. eds. Virology Monographs 18. Wien: Springer Verlag; 1981.

Dalmat, H.T. Arthropod Transmission of Rabbit Fibromatosis (Shope). Journal of Hygiene (Cambridge) 57: 1-29; 1959.

Desmettre, P.; Languet, B.; Chappuis, G.; Brochier, B.; Thomas, I.; Lecocq, J.-P.; Kieny, M.-P.; Blancou, J.; Aubert, M.; Artois, M.; Pastoret, P.-P. Use of Vaccinia Rabies Recombinant for Oral Vaccination of Wildlife. Veterinary Microbiology 23: 227-236; 1990.

Dufour, B.; Aubert, M.; Bonnel, A.; Toma, B. Lutte contre la rage bovine en France en 1987: coût et bénéfice. Le Point Vétérinaire 21: 523-528; 1989.

Dumbell, K.R. Aspects of the Biology of Orthopoxviruses Relevant to the Use of Recombinant Vaccinias as Field Vaccines. In: Quinnan, G.V., ed. Vaccinia Viruses as Vectors for Vaccine Antigens. New York: Elsevier; 1985: 9-13.

Dumbell, K.R.; Bedson, H.S. The use of ceiling temperature and reactivation in the isolation of pox virus hybrids. Journal of Hygiene (Cambridge) 62: 133-140; 1964.

Ehrengut, W.; Sarateanu, D.E.; Alswede, U.; Habib, A.; Tetzlaff, G. Vaccinia Virus Variants as Presumable Cause of Vaccinial Complications. Archives of Virology 48: 229-238; 1975.

Evans, D.H.; Stuart, D.; McFadden, G. High Levels of Genetic Recombination among Cotransfected Plasmid DNAs in Poxvirus-Infected Mammalian Cells. Journal of Virology 62: 367-375; 1988.

Fenner, F. Vaccinia Virus as a Vaccine, and Poxvirus Pathogenesis. In: Binns, M.M.; Smith, G.L., eds. Recombinant Poxviruses. Boca Raton: CRC Press; 1992: p. 1-43.

Fenner, F.; Comben, B.M. Genetic Studies with Mammalian Poxviruses. I. Demonstration of Recombination between Two Strains of Vaccinia Virus. Virology 5: 530-548; 1958.

Fenner, F.; Ratcliffe, F.N. Myxomatosis. Cambridge: Cambridge University Press; 1965.

Fenner, F.; Wittek, R.; Dumbell, K.R. The Orthopoxviruses. London: Academic Press; 1989. Fox, J.L. Three recombinant vaccine tests stir debate. Bio/Technology 5: 13-14; 1987a.

Fox, J.L. Public opinion: sense vs. sensibility. Bio/Technology 5: 14; 1987b.

Gershon, P.D.; Kitching, R.P.; Hammond, J.M.; Black, D.N. Poxvirus Genetic Recombination during Natural Virus Transmission. J. Gen. Virol. 70: 485-489; 1989.

Glosser, J.W. Environmental assessment and preliminary finding of no significant impact. United States Department of Agriculture, Animal and Plant Health Inspection Service (APHIS). 1989. (In: Rhône Mérieux. Raboral V-RG. N 175/0-3, Décembre 1992. Dossier innocuité, Réferences)

Goebel, S.J.; Johnson, G.P.; Perkus, M.E.; Davis, S.W.; Winslow, J.P.; Paoletti, E. The Complete DNA Sequence of Vaccinia Virus. Virology 179: 247-266; 1990.

Grinsted, J. Risk Assessment and Contained Use of Genetically Modified Microorganisms (GMMs). In: Tzotzos, G.T. Genetically Modified Organisms. A Guide to Biosafety. Wallingford: CAB International; 1995:p. 17-35.

Guillaume, J.C.; Saiag, P.; Wechsler, J.; Lescs, M.C.; Roujeau, J.C. Vaccinia from recombinant virus expressing HIV genes. Lancet 337: 1034-1035; 1991.

Gurvich, E.B.; Vilesova, I.S. Vaccinia Virus in Postvaccinal Encephalitis. Acta Virologica 27: 154-159; 1983.

Hahn, E.C. Safety of Recombinant Vaccines. In: Isaacson, R.E. ed. Recombinant DNA Vaccines: Rationale and Strategy. New York: Dekker; 1992: p. 387-400.

Henderson, L.M.; Katz, J.B.; Erickson, G.A.; Mayfield, J.E. In vivo and in vitro genetic recombination between conventional and gene-deleted vaccine strains of pseudorabies virus. Am. J. Vet. Res. 51: 1656-1662; 1990.

Henning, K.; Czerny, C.-P.; Meyer, H.; Müller, T.; Kramer, M. A seroepidemiological survey for orthopox virus in the red fox (*Vulpes vulpes*). Veterinary Microbiology 43: 251-259; 1995.

Javier, R.T.; Sedarti, F.; Stevens, J.G. Two Avirulent Herpes Simplex Viruses Generate Lethal Recombinants in Vivo. Science 234: 746-748; 1986.

Jones, L.; Ristow, S.; Yilma, T.; Moss, B. Accidental human vaccination with vaccinia virus expressing nucleoprotein gene. Nature 319: 543; 1986.

Kaplan, C. Vaccinia virus: a suitable vehicle for recombinant vaccines?. Arch. Virol. 106: 127-139; 1989.

Kaplan, C.; Healing, T.D.; Evans, N.; Healing, L.; Prior, A. Evidence of Infection by Viruses in Small British Field Rodents. Journal of Hygiene (Cambridge) 84: 285-294; 1980.

Karupiah, G.; Coupar, B.; Ramshaw, I.; Boyle, D.; Blanden, R.; Andrew, M. Vaccinia Virus-Mediated Damage of Murine Ovaries and Protection by Virus-Expressed Interleukin-2. Immunol. Cell Biol. 68: 325-333; 1990.

Kieny, M.P.; Lathe, R.; Drillien, R.; Spehner, D.; Skory, S.; Schmitt, D.; Wiktor, T.; Koprowski, H.; Lecocq, J.P. Expression of rabies virus glycoprotein from a recombinant vaccinia virus. Nature 312: 163-166; 1984.

Koprowski, H. Rabies Oral Immunizaton. Current Topics in Microbiology and Immunology 146: 137-151; 1989.

Kurata, T.; Aoyama, Y.; Kitamura, T. Demonstration of Vaccinia Virus Antigen in Brains of Postvaccinal Encephalitis Cases. Japan. J. Med. Sci. Biol. 30: 137-147; 1977.

Lal, S.M.; Singh, I.P. Buffalopox - A Review. Trop. Anim. Hlth Prod. 9: 107-112; 1977.

Lawson, K.F.; Hertler, R.; Charlton, K.M.; Campbell, J.B.; Rhodes, A.J. Safety and Immunogenicity of ERA strain of Rabies Virus Propagated in a BHK-21 Cell Line. Canadian Journal of Veterinary Research 53: 438-444; 1989.

Levine, M.M. Non-Target Effects of Live Vaccines: Myth, Reality and Demagoguery. In: Brown, F. ed. Non-target Effects of Live Vaccines. Developments in Biological Standardisation 84. Basel: Karger; 1995: p. 33-38.

Lourie, B.; Bingham, P.G.; Evans, H.H.; Foster, S.O.; Nakano, J.H.; Herrmann, K.L. Human infection with monkeypox virus: laboratory investigation of six cases in West Africa. Bulletin WHO 46: 633-639; 1972.

Lourie, B.; Nakano, J.H.; Kemp, G.E.; Setzer, H.W. Isolation of Poxvirus from an African Rodent. The Journal of Infectious Diseases 132: 677-681; 1975.

Mackett, M. Vaccinia Virus as a Cloning and Delivery System for Vaccines. In: Isaacson, R. E., ed. Recombinant DNA Vaccines: Rationale and Strategy. New York: Dekker; 1992: p. 223-264.

Mackett, M. Vaccinia virus as a vector for delivering foreign antigens. Virology 1: 39-47; 1990.

Marennikova, S.S.; Maltseva, N.N.; Korneeva, V.I.; Garanina, N.M. Outbreak of Pox Disease among Carnivora (Felidae) and Edentata. The Journal of Infectious Diseases 135: 358-366; 1977.

Martin, W.B.; Scott, F.M.M.; Lauder, I.M.; Nash, A. Poxvirus Infection of Cats. Veterinary Record 115: 36; 1984.

Masson, E.; Aubert, M.; Rotivel, Y. Impact des campagnes de vaccination des renards contre la rage en France sur le ramassage des appâts-vaccins et la contamination des personnes. 25 mars 1994: Compte-rendu de la deuxième réunion des centres antirabiques; 1994; p. 33-53.

McFadden, G. Rabbit, Hare, Squirrel and Swine Poxviruses. In: Webster, R.G.; Granoff, A., eds. Encyclopedia of Virology. London: Academic Press; 1994: p. 1153-1160.

McNally, R. Genetic Madness. The European Rabies Eradication Programme. The Ecologist 24: 207-212; 1994.

Merchlinsky, M. Intramolecular Homologous Recombination in Cells Infected with Temperature - Sensitive Mutants of Vaccinia Virus. Journal of Virology 63: 2030-2035; 1989.

Mohr, W.; Schumacher, H.-H.; Weyer, F. eds. Lehrbuch der Tropenkrankheiten. Stuttgart: Georg Thieme Verlag; 1975: p. 303-304.

Moss, B. Molecular Biology of Poxviruses. In: Binns, M.M.; Smith, G.L., eds. Recombinant Poxviruses. Boca Raton: CRC Press; 1992: p. 45-80.

Myers, K. Studies in the epidemiology of infectious myxomatosis of rabbits. II. Field experiments. August-November 1950, and the first epizootic of myxomatosis in the riverine plain of south-eastern Australia . Journal of Hygiene (Cambridge) 52: 47-59; 1954.

OECD; International Association of Standardization. Summary Report. In: Brown, F. ed. Non-Target Effects of Live Vaccines. Developments in Biological Standardisation 84. Basel: Karger; 1995: p. 3-9.

Openshaw, P.J.M.; Alwan, W.H.; Cherrie, A.H.; Record, F.M. Accidental infection of laboratory worker with recombinant vaccinia virus. Lancet 338: 459; 1991.

Opgenorth, A.; Strayer, D.; Upton, C.; McFadden, G. Deletion of the Growth Factor Gene Related to EGF and TGF Reduces Virulence of Malignant Rabbit Fibroma Virus. Virology 186: 175-191; 1992.

Palca, J. Row over Vaccine Trial. Nature 331: 470; 1988.

Pastoret, P.P.; Brochier, B.; Boulanger, D. Target and Non-Target Effects of a Recombinant Vaccinia-Rabies Virus Developed for Fox Vaccination against Rabies. In: Brown, F. ed. Non-Target Effects of Live Vaccines. Developments in Biological Standardisation 84. Basel: Karger; 1995: p. 183-193.

Pastoret, P.-P.; Brochier, B.; Blancou, J.; Artois, M.; Aubert, M.; Kieny, M.-P.; Lecocq, J.-P.; Languet, B.; Chappuis, G.; Desmettre, P. Development and deliberate release of a vaccinia rabies recombinant virus for the oral vaccination of foxes against rabies. In: Binns, M.M.; Smith, G. L., eds. Recombinant Poxviruses. Boca Raton: CRC Press; 1992a: p. 163-206.

Pastoret, P.P.; Brochier, B. Development of a Recombinant Vaccinia-Rabies Vaccine for Oral Vaccination of Foxes against Rabies. Develop. biol. Standard. 79: 105-111; 1992b.

Pépin, M.; Blancou, J.; Aubert, M.F.A.; Barrat, J.; Coulon, P.; Flamand, A. Oral Immunization against Rabies with an Avirulent Mutant of the CVS Strain: Evaluation of its Efficacy in Fox (*Vulpes vulpes*) and its Infectivity in Seven Other Species. Ann. Inst. Pasteur / Virol. 136 E: 65-73; 1985.

Pickup, D.J.; Ink, B.S.; Parsons, B.L.; Hu, W.; Joklik, W.K. Spontaneous deletions and duplications of sequences in the genome of cowpox virus. Proc. Natl. Acad. Sci. USA 81: 6817-6821; 1984.

Polak, M.F. Complications of Smallpox Vaccination in the Netherlands, 1959-1970. In: Regamey, R.H.; Cohen, H. eds. International Symposium on Smallpox Vaccine, 11-13 October 1972, Bilthoven. Symposia Series in Immunobiological Standardization 19: 235-242. Basel: Karger; 1972.

Polak, M.F.; Beunders, B.J.W.; VanDerWerff, A.R.; Sanders, E.W.; VanKlaveren, J.N.; Brans, L.M. A Comparative Study of Clinical Reaction Observed after Application of Several Smallpox Vaccines in Primary Vaccination of Young Adults. Bulletin WHO (Bull. Org. mond. Santé) 29: 311-322; 1963.

Quinnan, G.V. ed. Vaccinia Viruses as Vectors for Vaccine Antigens. Nov. 13-14 1984: Proceedings of the Workshop on Vaccinia Viruses as Vectors for Vaccine Antigens, Chevy Chase. New York: Elsevier; 1985.

Redfield, R.R.; Wright, D.C.; James, W.D.; Jones, T.S.; Brown, C.; Burke, D.S. Disseminated Vaccinia in a Military Recruit with Human Immunodeficiency Virus (HIV) Disease. The New England Journal of Medicine 316: 673-676; 1987.

Robert Koch - Institut des Bundesgesundheitsamtes, Abteilung Biologische Sicherheit / Gentechnik. H.-J. Buhk. Bericht betreffend Raboral V-RG zuhanden der Commission of the European Communities; 1993.

Rodriguez, D.; Rodriguez, J.-R.; Rodriguez , J.F.; Trauber, D. Highly attenuated vaccinia virus mutants for the generation of safe recombinant viruses. Proc. Natl. Sci. USA 86: 1287-1291; 1989.

Rupprecht, C.E.; Hanlon, C.A.; Niezgoda, M.;Buchanan J.R.; Diehl, D.; Koprowski, H. Recombinant rabies vaccines: efficacy assessment in free-ranging animals. Onderstepoort Journal of Veterinary Research 60: 463-468; 1993.

Rupprecht, C.E.; Hanlon, C.A.; Koprowski, H.; Hamir, A.N. Development of a Recombinant Rabies Vaccine: Safety Implications. Presented at the 57th annual North American Wildlife and Natural Resource Conference; 1992a.

Rupprecht, C.E.; Hanlon, C.A.; Cummins, L.B.; Koprowski, H. Primate responses to a vaccinia-rabies glycoprotein recombinant virus vaccine. Vaccine 10: 368-374; 1992b.

Rupprecht, C.E.; Hamir, A.N.; Johnston, D.H.; Koprowski, H. Efficacy of a Vaccinia-Rabies Glycoprotein Recombinant Virus Vaccine in Raccoons (*Procyon lotor*). Reviews of Infectious Diseases 10: S803-S809; 1988.

Rupprecht, C.E.; Wiktor, T.J.; Johnston, D.H.; Hamir, A.N.; Dietzschold, B.; Wunner, W.H.; Glickman, L.T.; Koprowski, H. Oral immunization and protection of racoons (Procyon lotor) with a vaccinia-rabies glycoprotein recombinant virus vaccine. Proc. Natl. Acad. Sci. USA 83: 7947-7950; 1986.

Sineriz, F.; La Torre, J.L. Round Table 5: Case histories of deliberate release. In: Sussman, M.; Collins, C.H. eds. The release of genetically-engineered microorganisms. 1988: p. 253-263.

Smith, J.S.; Orciari, L.A.; Yager, P.A. Molecular epidemiology of rabies in the United States. Virology 6: 387-400; 1995.

Spyropoulos, D.D.; Roberts, B.E.; Panicali, D.L.; Cohen, L.K. Delineation of the Viral Products of Recombination in Vaccinia Virus - Infected Cells. Journal of Virology 62: 1046-1054; 1988.

Steck, F.; Wandeler, A.; Bichsel, P.; Capt, S.; Häfliger, U.; Schneider, L. Oral Immunization of Foxes against Rabies. Laboratory and Field Studies. Comp. Immun. Microbiol. Infect. Dis. 5: 165-171; 1982.

Stöhr, K. Personal Communication, 14.1.1997. Source: WHO Data Bank on Oral Immunization of Foxes against Rabies.

Störfallverordnung. Handbuch II zur Störfallverordnung. Richtlinien für Betriebe mit Mikroorganismen. Bern: EDMZ; 1992.

Strayer, D.S.; Cabirac, G.; Sell, S.; Leibowitz, J.L. Malignant Rabbit Fibroma Virus: Observations on the Culture and Histopathologic Characteristics of a New Virus-Induced Rabbit Tumor. J. Natl Cancer Inst. 71: 91-104; 1983a.

Strayer, D.S.; Sell, S. Immunohistology of Malignant Rabbit Fibroma Virus - a Comparative Study with Rabbit Myxoma Virus. JNCI 71: 105-116; 1983b.

Strayer, D.S.; Sell, S.; Skaletsky, E.; Leibowitz, J.L. Immunologic Dysfunction during Viral Oncogenesis. I. Nonspecific Immunosuppression Caused by Malignant Rabbit Fibroma Virus. Journal of Immunology 131: 2595-2600; 1983c.

Strayer, D.S.; Skaletsky, E.; Cabirac, G.F.; Sharp, P.A.; Corbeil, L.B.; Sell, S.; Leibowitz, J.L. Malignant Rabbit Fibroma Virus Causes Secondary Immunosuppression in Rabbits. The Journal of Immunology 130: 399-404; 1983d.

Strayer, D.S.; Skaletsky, E.; Leibowitz, J.L. In Vitro Growth of Two Related Leporipoxviruses in Lymphoid Cells. Virology 145: 330-334; 1985.

Tartaglia, J.; Pincus, S.; Paoletti, E. Poxvirus-Based Vectors as Vaccine Candidates. Critical Reviews in Immunology 10: 13-30; 1990.

Thomas, I.; Brochier, B.; Languet, B.; Blancou, J.; Perharpre, D.; Kieny, M.P; Desmettre, P.; Chappuis, G.; Pastoret, P.-P. Primary multiplication site of the vaccinia-rabies glycoprotein recombinant virus administered to foxes by the oral route. J. Gen. Virol. 71: 37-42; 1990.

Thomsett, L.R.; Baxby, D.; Denham, E.M.H. Cowpox in the domestic cat. Veterinary Record 108: 567; 1978.

Tiedje, J.M.; Colwell, R.K.; Grossman, Y.L.; Hodson, R.E.; Lenski, R.E.; Mack, R.N.; Regal, P.J. The planned introduction of genetically engineered organisms: Ecological considerations and recommendations. Ecology 70: 298-315; 1989.

Tillotson, J.R.; Axelrod, D.; Lyman, D.O. Rabies in a Laboratory Worker - New York. Morbidity and Mortality Weekly Report June 3: 183-184; 1977.

Tuffereau, C.; Leblois, H.; Bénéjean, J.; Coulon, P.; Lafay, F.; Flamand, A. Arginine or Lysine in Position 333 of ERA and CVS Glycoprotein Is Necessary for Rabies Virulence in Adult Mice. Virology 172: 206-212; 1989.

Uhaa, I.J.; Dato, V.M.; Sorhage, F.E.; Beckley, J.W.; Roscoe, D.E.; Gorsky, R.D.; Fishbein, D.B. Benefits and costs of using an orally absorbed vaccine to control rabies in raccoons. JAVMA 201: 1873-1882; 1992.

Upton, C.; Macen, J.L.; Maranchuk, RA.; DeLange, A.M.; McFadden, G. Tumorigenic Poxviruses: Fine Analysis of the Recombination Junctions in Malignant Rabbit Fibroma Virus, a Recombinant between Shope Fibroma Virus and Myxoma Virus. Virology 166: 229-239; 1988.

Upton, C.; McFadden, G. Tumorigenic Poxviruses: Analysis of Viral DNA Sequences Implicated in the Tumorigenicity of Shope Fibroma Virus and Malignant Rabbit Virus. Virology 152: 308-321; 1986.

Wachendörfer, G.; Farrenkopf, R.; Lohrbach, W.; Förster, U.; Frost, J.W.; Valder, W.A. Passageversuche mit einer Varianten des Tollwutimpfstammes ERA bei wildlebenden Spezies (Ondatra zibethica und Rattus norvegicus) - Ein Beitrag zur oralen Immunisierung von Füchsen gegen Tollwut. Deutsche Tierärztliche Wochenschrift 85: 273-308; 1978.

Wandeler, A.I. Oral Immunization of Wildlife. In: Baer, G.M. ed. The Natural History of Rabies. Boca Raton: CRC Press; 1991:p. 485-503.

Wandeler, A.I. Control of wildlife rabies: Europe. In: Campbell, J.B.; Charlton, K.M: eds. Rabies. Boston: Kluwer Academic Publishers; 1988a.

Wandeler, A.I.; Capt, S.; Kappeler, A.; Hauser, R. Oral immunization of wild life against rabies: concept and first field experiments.. Rev infect Dis 10: 649-653; 1988b.

WHO. Report of the Symposium on Rabies Control in Asian Countries. Jakarta, Indonesia, 27-30 April 1993. WHO/Rab.Res./93.44: 1-42; 1993a.

WHO. Report of the Fourth WHO Consultation on Oral Immunization of Dogs against Rabies. Geneva, 14-15 June 1993. WHO/ Rab.Res./93.42:1-17; 1993b.

WHO. 3rd Consultation on Oral Immunization of Dogs against Rabies: Organized by WHO with the participation of the Office International des Epizooties. WHO/Rab.Res./92.38: 1-14; 1992.

WHO. Potential use of live viral and bacterial vectors for vaccines. Vaccine 8: 425-437; 1990.

Wiktor, T.J.; MacFarlan, R.I.; Dietzschold, B.; Rupprecht, C.; Wunner, W.H. Immunogenic Properties of Vaccinia Recombinant VirusExpressing the Rabies Glycoprotein. Ann. Inst. Pasteur / Virol. 136 E: 105-111; 1985.

Winkler, W.G.; Shaddock, J.H.; Williams, L.W. Oral rabies vaccine: Evaluation of its infectivity in three species of rodents. Am. J. Epidemiol. 104: 294-298; 1976.

Wittek, R. Vaccinia Virus. In: Webster, R.G.; Granoff, A., eds. Encyclopedia of Virology. Volume 3. London: Academic Press; 1994: p. 1507-1513.

Wittek, R.; Menna, A.; Schümperli, D.; Stoffel, S.; Müller, H.K.; Wyler, R. *Hin*dIII and *Sst* I Restriction Sites Mapped on Rabbit Poxvirus and Vaccinia Virus DNA. Journal of Virology 23: 669-678; 1977.

Yilma, T. Genetically engineered vaccines for animal viral diseases. Journal of the American Veterinary Medical Association 204: 1606-1615; 1994.

Yilma, T. Vaccinia Virus Recombinant Vaccines for Rinderpest . In: Brown, F. ed. Non-Target Effects of Live Vaccines. Developments in Biological Standardisation 84. Basel: Karger; 1995: p. 201-208.

Zanoni, R.; Breitenmoser, U.; Peterhans, E. Mit Gentechnologie gegen die Tollwut. Experten über eine Alternative zum herkömmlichen Impfstoff. Neue Zürcher Zeitung, 16 April 1996.