Aus dem *Laboratoire des Lyssavirus*
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Towards a vaccine against the European Lyssaviruses - a structural and immunological approach

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Abbreviations

aa	aminoacid
ADCC	antibody dependent cell cytotoxicity
APC	antigen presenting cell
CFA	complete Freund's adjuvant
cpm	counts per minute
CTL	cytotoxic T lymphocytes
CVS	challenge virus strain
EBL	European bat lyssavirus
ERA	Evelyn-Rokitmicki-Abelseth
ERIG	equine rabies immunoglobulin
FITC	fetal calf serum
FITC	fluoresceine isothiocyanate
FFU	fluorescent focus units
G	glycoprotein
G-Bac	recombinant glycoprotein expressed in G-recombinant baculovirus infected cells
G-Vacc	G-recombinant vaccinia virus
Gs-Bac	recombinant glycoprotein secreted by G-recombinant baculovirus infected cells
HRIG	human rabies immunoglobulin
IFN	interferon
IL	interleukin
IPRV	inactivated purified rabies virus
IU	international units
L	Rabies polymerase
M	matrix protein
MHC	major histocompatibility complex
Mok	Mokola
mono	monoclonal
N	nucleoprotein
N-Bac	recombinant glycoprotein expressed in N-recombinant baculovirus infected cells
N-Vacc	N-recombinant vaccinia virus
NK	natural killers
NT	Na-Tris-buffer
OGP	octylglycopyranoside
P	phosphoprotein
PBS	phosphate buffer saline
PFU	plaque forming unit
PM	Pitman Moore
poly	polyclonal
PV	Pasteur virus
RNP	ribonucleoprotein
rpm	rotations per minute
TBE	tris borate EDTA buffer
Th	T helper
U	unit
VNA	virus neutralizing antibodies
VSV	vesicular stomatitis virus

The following parts of the results shown have been published in advance:

"Production of Lyssavirus proteins in the baculovirus expression system and their use as potential vaccine"
A. Drings, B. Chambert, C.Jallet, N.Tordo, P.Perrin and Y.Jacob
M. J. T. Carrondo et al. (eds.), Animal Cell Technology, 191-195, 1997

"Importance of the rabies virus glycoprotein antigenic site III and transmembrane domain for presenting foreign epitopes: use of DNA-based immunization"
C. Bahloul, A. Drings, Y. Jacob, P. Bouige, C. Jallet, N. Tordo, P. Perrin
ESVV, 4th International Congress of Veterinary Virology, Edinburgh, Scotland, 24.-27. August 1997
(Abstract)

Content

INTRODUCTION

I. INTRODUCTION

I. 1. Rabies and lyssaviruses

I. 1. 1. General considerations

Rabies has been known for more than 20000 years. The first description dates from the 23rd century before Christ in the Mesopotamian *Laws of Eshnuma* (Fu, 1997). Over the centuries, this encephalitis in warm-blooded animals and humans has been recognized to be an always lethal disease. It was only in the end of the 19th century that the first vaccine against rabies was developed (Pasteur, 1885), till today the only treatment after exposure to a rabid animal. In spite of numerous efforts 35000-50000 human fatal cases per year are reported (WHO, 1994), real numbers certainly being much higher. This makes rabies one of the 10 most mortal infectious diseases. In addition to those still unsolved problems, emerging rabies-related viruses have been recently described (Fraser et al., 1996), leaving no doubt about the importance of rabies research.

I. 1. 2. Clinical manifestations

In humans the incubation period of rabies usually varies between 31 to 90 days with extreme cases of less than a week or more than a year (for review see Baer et al., 1990a). The length of incubation period depends on the innervation of the bite site, proximity of the bite site to the CNS, severity of the bite, quantity of the virus inoculated and age and immune status of the host. It is only during this period that anti-rabies treatment by vaccination is possible (see § I. 2. 1.). The clinical phase begins with mild and non-specific symptoms (prodromal period), involving constitutional changes and functional modification of the respiratory and gastrointestinal apparatus and of the central nervous system (CNS). The only specific symptom that is often noted is pain or paresthesia at the bite site. During the acute neurologic period either hyperactivity or paralysis are predominant (furious or paralytic rabies). Among several less specific symptoms such as agitation and hallucinations, hydrophobia is the pathognomic sign of rabies, due to an exaggerated respiratory reflex. In paralytic rabies that appear most frequently after exposure to bat virus strains, hydrophobia only occurs terminally or lacks completely. Furious rabies ends with coma after 2-7 days, whereas paralytic rabies lasts up to 30 days. After transition to coma, respiratory arrest occurs within hours or days.

I. 1. 3. Rabies pathology

I. 1. 3. a. Virus propagation *in vivo*

The way of viral propagation towards the CNS after inoculation by bite has not been clearly demonstrated yet (for review see Murphy, 1977; Tsiang, 1993; Charlton, 1994). Rabies virus seems to go through a first cycle of replication in the striated muscle cells, where the virus is in a state of sequestration during the incubation time (Murphy et al., 1973; Murphy and Bauer, 1974). It has been suggested that the infection of muscle cells is mediated by the nicotine acetylcholine receptor (Lentz et al., 1981; Baer et al., 1990b).

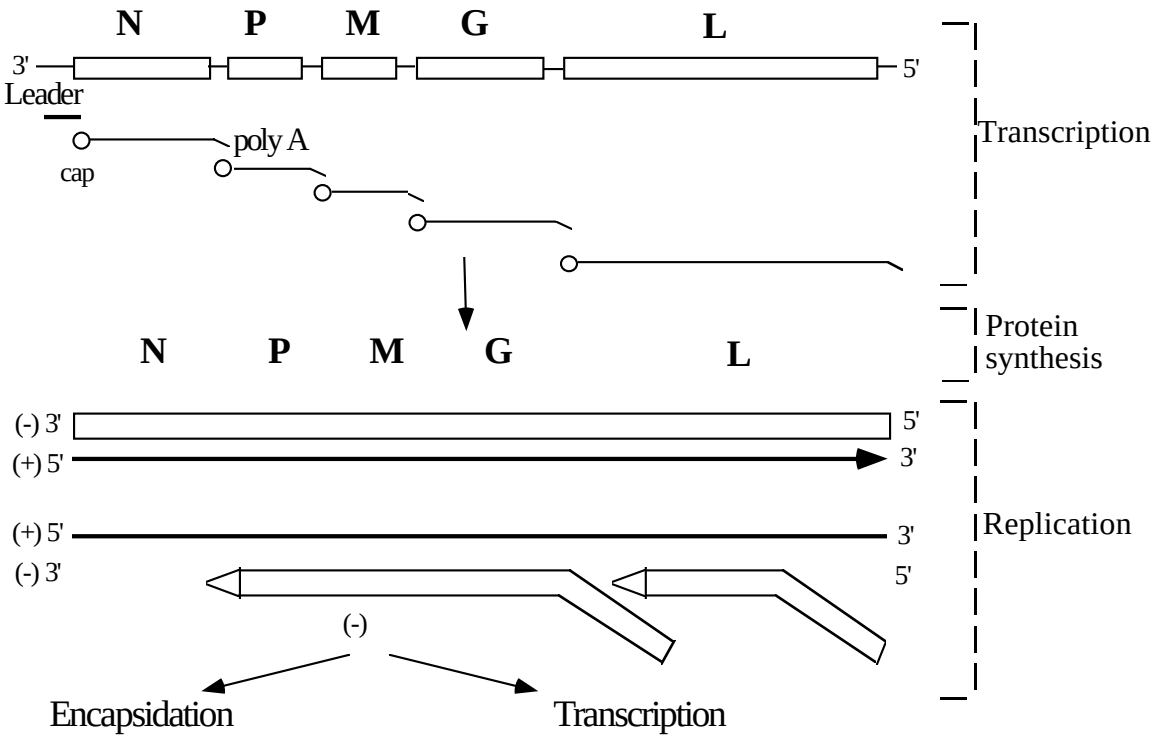
For further spread, the virus accumulates at neuromuscular and neurotendinal spindles and at the neuromuscular junctions and enters the nervous system through unmyelinated sensory and motor terminals. In some cases, viral entry has been reported without prior replication at the inoculation site (Shankar et al., 1991). It is important to note, that once rabies virus has entered the nervous system it is no longer accessible to anti-rabies antibodies (Murphy and Bauer, 1974). The virus reaches the spinal cord by centripetal spread (axoplasmic flow of 12 to 24 mm per day) after about 60 hours. It is subsequently transported within hours to the brainstem (Murphy et al., 1973), where the infection of the limbic system induces changes of behaviour including alertness and loss of natural timidity.

After infection of the brain, the virus takes again the axoplasmic route for centrifugal movement, which leads to infection of the periphery, notably of the salivary gland mucous epithelium and thus to viral shed into the saliva.

I. 1. 3. b. Virus replication *in vitro*

In vitro, infection of fibroblasts occurs after fixation of rabies G on a cellular receptor that seems to be constituted of lipids, lipoproteins and/or lipopolysaccharides (Perrin et al., 1982; Superti et al., 1986). After adsorption on the cellular receptor, the virus enters by endocytosis (Perrin et al., 1982; Superti et al., 1984; Gaudin et al., 1993) and under the reduction of pH in the endocytotic vesicle the viral envelope fuses with the vesicular membrane releasing the ribonucleoprotein complex (RNP) into the cytoplasm.

The mechanism of viral replication is not yet fully understood. However, according to the postulated strategy (Tordo et al., 1986a; Tordo and Poch, 1988), viral replication (figure. I. 1.) takes place in the cytoplasm and starts at the 3' end with the transcription of the leader RNA and the mRNAs coding for the viral proteins. At the same time, some full length copies of the viral positive RNA are transcribed. The transcription is less and less efficient with rising distance from the 3' end: the gene of the nucleoprotein (N) is therefore the most frequently transcribed. The newly synthesized N encapsulates the nascent RNA simultaneously



N	=	Nucleoprotein
P	=	Phosphoprotein
M	=	Matrix protein
G	=	Glycoprotein
L	=	Polymerase

Figure I. 1. Rabies virus replication.

to transcription. This encapsidation seems to induce the switch from transcription to replication. The full length positive stranded RNA now serves as a template for negative stranded viral RNA synthesis and encapsidation with the N protein. The structural proteins assemble with the RNP about 24h after infection and viral particles leave the host cell by budding from the cell membrane (Hummeler et al.,1967).

I. 1. 3. c. Virus pathogenicity

The pathogenicity of rabies viruses seems to depend to a certain extend on the presence of an arginine or a lysine residue at the position 333 within the antigenic site III (see § I. 1. 7. a.) of the glycoprotein: the avirulent rabies virus mutants which have been isolated do not contain these amino-acid residues at this position (Dietzschold et al., 1983b; Seif et al., 1985; Tuffereau et al., 1989). Moreover, two genotypes of the lyssaviruses, Mokola and Lagos bat (see § 1.1.4) which carry neither arginine nor lysine at position 333 of the glycoprotein (Badrane, 1997) are not pathogenic after intramuscular injection (Perrin et al., 1996b). Beside the site III, the site II of the glycoprotein is discussed to play a role in the rabies virus pathogenicity as mutations on the aminoacid (aa) 198 induce modifications of pathogenicity (Prehaud et al., 1988).

I. 1. 4. Classification and epidemiology

Rabies and rabies-related viruses belong to the lyssavirus genus which together with the vesiculovirus and ephemerovirus genera forms the family of the rhabdoviruses. Rhabdoviruses are members of the Mononegavirales order, which includes as well the Paramyxoviruses and the Filoviruses (Wildy, 1971).

Lyssaviruses have been divided into four serotypes according to their antigenic properties (Schneider et al., 1973; Flamand et al., 1980; Wiktor et al., 1980) and into 6 genotypes according to their genomic sequence (Bourhy et al., 1993) (table I. 2.). More recently, it has been shown that two groups of lyssaviruses can be distinguished by the cross-reactivity of neutralizing antibodies against different lyssaviruses: genotypes 1, 4, 5 and 6 belong to the group "Lyssa-1" and genotypes 2 and 3 to the group "Lyssa-2" (Bahloul et al., 1997).

The genotype 1 contains the classical rabies viruses and is found worldwide except for some isolated regions. The vaccinal strains belong to this genotype as they have derived from classical rabies viruses. The genotypes 2 (prototype: Lagos-bat virus), 3 (prototype: Mokola virus) and 4 (prototype: Duvenhage virus) have all been isolated on the African continent in insectivorous bats, small rodents, dogs, cats and in some cases in man. The genotypes 5 and 6 represent the EBL1 and 2

GENOTYPE	SEROTYPE	CROSS-REACTIVE GROUP	DISTRIBUTION	ANIMAL SPECIES
1. Rabies	1	Lyssa 1	all over the world except: Australia (?), UK, Ireland, New Zealand, Japan, Antarctica, Scandinavia, Hawaii	man, domestic and wild carnivores, herbivores, bats
2. Lagos-bat	2	Lyssa 2	Africa	frugivorous bats, cats, dogs
3. Mokola	3	Lyssa 2	Africa	man, cats, dogs, rodents
4. Duvenhage	4	Lyssa 2	Africa	man, insectivorous bats
5. EBL1	1 or 4 ?	Lyssa 1	Europe	man, insectivorous bats: Epseticus
6. EBL2	1 ?	Lyssa 1	Europe (Switzerland, Netherlands)	man, insectivorous bats: Myotis
7. Pteroid Lyssavirus (PLV)	?	Lyssa 1 ?	Australia	man, frugivorous bats (flying foxes)

Table I. 2. Classification and distribution of the lyssaviruses (Bourhy et al., 1993 ; Fraser et al., 1996; Badrane et al., 1997; Bahloul et al., 1997).

(European Bat Lyssaviruses) viruses which have been isolated in insectivorous bats in Europe (EBL 1: Epseticus serotinus, EBL 2: Myotis; Bourhy et al., 1992; Grauballe, 1987; Schneider, 1982). The European bat lyssaviruses have been as well responsible for human deaths (Lumio et al., 1986; Mollgaard, 1985; Selimov, 1991). In 1996 one positive bat was diagnosed in the United Kingdom (Whitby et al., 1996) which is considered to be exempt of rabies.

The genotypes 5 and 6 show much less variation than genotype 1 (Kissi et al., 1995) what might indicate a close adaptation to the host. Following the different isolations of genotype 5 (figure I. 3.), it has been suggested that EBL1 came to Europe from two different directions, one part from North Africa (EBL1b) and another from the East (EBL1a) and that they mixed in the Netherlands. The epidemiological data for EBL2 are not yet sufficient to yield a hypothesis about its origin (Amengual et al., 1997). Recently, another lyssavirus has been isolated in Australian frugivorous bats (flying foxes; Pteropus alecto and Pteropus scamulatus). It has been responsible for a fatal encephalitis in a woman. The new lyssavirus is discussed to be classified in a new genotype (genotype 7) even though it is closely related to genotype 1 (Fraser et al., 1996; Ban, 1997).

It is important to note that terrestrial (mainly carnivores) and aerial vectors (bats) of lyssaviruses can be distinguished. Indeed, if it is possible to attempt vaccination of terrestrial vectors, no valuable solution is reported for aerial vector vaccination. Thus after massive campaigns for oral immunization of foxes terrestrial rabies might disappear in Europe whereas aerial rabies will remain, creating a similar situation as in regions that are exempt of terrestrial rabies but which can possess aerial lyssavirus vectors (e.g. Great Britain or Australia). When one looks closer at the importance of bats as lyssavirus vectors it appears as a striking fact that apart from Mokola, all lyssaviruses have been found in bats. Actually, epidemiological data have lead to the hypothesis that bats have been the ancestor reservoir of lyssaviruses capable to contaminate and to be adapted to terrestrial species (Badrane, 1997), indicating that the virus reservoir in bats deserves close attention.

I. 1. 5. Virus structure

I. 1. 5. a. Virus particle

The rabies virus particle (figure I. 4., p. 10) has a "bullet" shape with an average length and diameter of 180 nm and 75 nm respectively (for review see Wunner et al., 1988; Wunner, 1991). The helical ribonucleoprotein (RNP, a complex between the RNA, the viral polymerase, the nucleoprotein and the phosphoprotein - see § I. 2. 5. c.) with 30 to 35 coils is surrounded by a lipoprotein envelope. The viral envelope is about 7.5 to 10 nm thick and contains lipids of cellular origin as well as

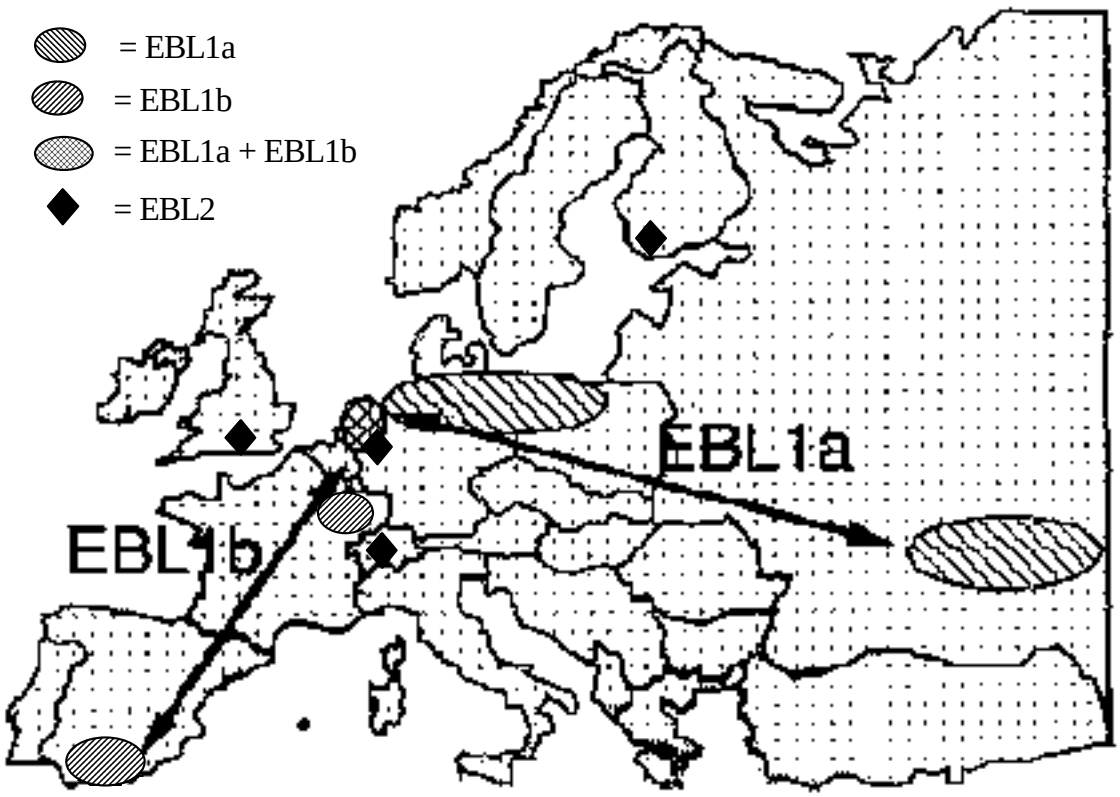
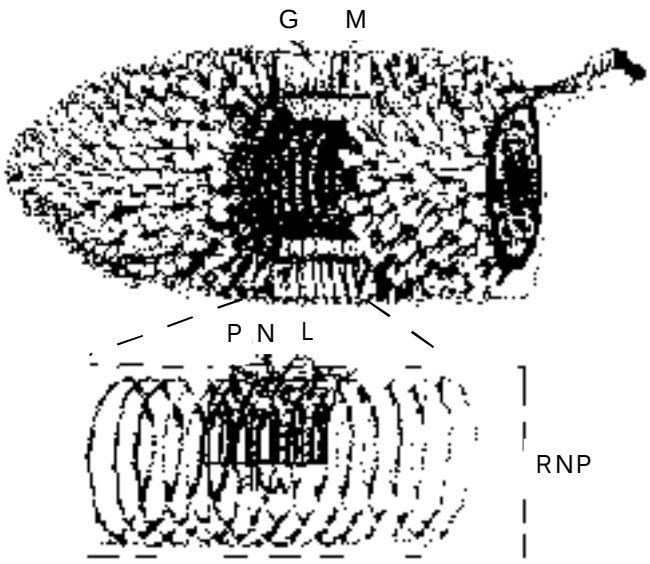
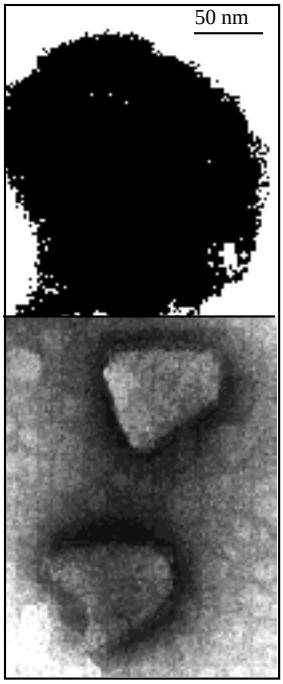


Figure I. 3.: Geographical distribution of EBL1 and EBL2 isolates in Europe (after H. Bourhy).

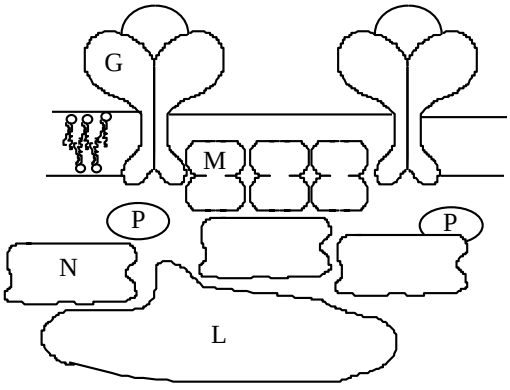
Figure I. 4. Structure and morphology of rabies virus.



(Wunner et al. 1988)



(P. Perrin)



(Delagneau et al. 1981, Perrin et al. 1987)

the viral glycoprotein and the matrix protein. The glycoprotein forms spike-like projections on the envelope and is associated with the matrix protein on the inner surface of the lipid bilayer (Delagneau et al., 1981). Apart from the cavity at the blunt end, the projections cover the surface of the virion tightly. The blunt end often has a slightly irregular shape, perhaps due to the budding from the plasma membrane of the infected cell.

I. 1. 5. b. Virus genome

The virus genome, which consists of single stranded, negative and thus non-infectious RNA, has a total length of 11,932 nucleotides and codes for a „leader sequence and 5 monocistronic mRNA transcripts for the viral proteins (Sokol et al., 1969; Tordo et al., 1986a): the nucleoprotein (1424 nucleotides), the phosphoprotein (991 nucleotides), the matrix protein (805 nucleotides), the glycoprotein (1674 nucleotides) and the viral polymerase (6475 nucleotides). Between the structural genes, intergenic sequences of different length have been described (Tordo et al., 1986b).

I. 1. 5. c. Virus proteins

NUCLEOPROTEIN (N)

N is a phoshorylated (aminoacid 389) protein of 450 amino acids (50.5 kDa) and present in the virion in a stochiometric amount of approximately 1800. It is tightly associated with the viral RNA protecting the RNA from ribonucleases and supposedly keeping it in a suitable configuration for transcription (Sokol and Clark, 1973b; Tordo et al., 1986a; Dietzschold et al., 1987a).

PHOSHOPROTEIN (P)

P is a phoshorylated protein of 297 amino acids (38 or 41 kDa) and present at about 950 molecules per virion. The phosphorylation is probably situated in the amino terminal half of the protein as in the case of P of the vesicular stomatitis virus (VSV; Weiss and Bennet, 1980). P is associated with the RNP (Chenik et al., 1994) but does not participate in the viral structure (Delagneau et al., 1981) - it has therefore also been denominated "non-structural" protein (NS). P is an important cofactor for the viral polymerase.

MATRIX PROTEIN (M)

About 1500 molecules of the M protein (202 amino acids, 23 kDa) are located on the inner surface of the viral envelope (Delagneau et al., 1981) where they interact with both the cytoplasmic domain of anchored G protein and the virus core, binding the membrane-associated (M and G) proteins to the RNP, possibly through interaction with N. M also plays a role in virus budding (Weiss and Bennet, 1980).

GLYCOPROTEIN (G)

Structure

The G protein is a glycosylated protein of 505 amino acids (65 kDa) which forms the viral spikes anchored in the lipid bilayer membrane. Each spike consists of a homopolymer of 3 molecules and extends 8.3 nm from the viral membrane (Delagneau et al., 1981; Gaudin et al., 1992). G accounts for about 30% of the total mass of viral proteins.

The amino acid sequence of the glycoproteins of the Evelyn-Rokitmicki-Abelseth (ERA), Challenge virus strain (CVS), Pasteur virus (PV) and HEP Flury strains have been deduced from the nucleotide sequence (Anilionis et al., 1981; Yelverton E., 1983; Tordo et al., 1986b; Morimoto K., 1989). G carries four potential N-glycosylation sites which are glycosylated to a different extend according to the virus strain. For example, G of the PV strain is not glycosylated at position 37 but glycosylated at position 158, 247 and 319 (see fig. I. 8. a., p. 22); Wunner et al., 1988) while on the G protein of CVS 2 or 3 of the potential glycosylation sites are glycosylated, resulting in two different protein sizes (Atanasiu et al., 1981; Wunner et al., 1985; Whitt et al., 1991).

Newly synthesized G (524 amino acids) contains 4 domains: 1) the cleavable aminoterminal signal peptide (19 hydrophobic aminoacids) responsible for translocation of the nascent protein across the RER membrane (Vishwanath et al., 1978; Lai and Dietzschold, 1981); 2) the ectodomain (438 aminoacids), as constituent of the viral spike responsible for virus cell interactions: infection, hemagglutination activity (Halonen et al., 1968) and immunological responses; 3) the transmembrane domain (22 hydrophobic aminoacids) responsible for the interaction with the membrane lipids; 4) the cytoplasmic domain (44 aminoacids) responsible for the interaction with core proteins.

Fusion activity

Depending on the pH, G can be present in at least three conformational forms which are in a pH-dependent equilibrium: 1) the "native" state detected at the viral surface above pH 7; 2) the "activated hydrophobic" state which interacts with the target membrane as a first step of the fusion process; 3) the "fusion-inactive" state which is present at low pH (Perrin and Atanasiu, 1981; Gaudin and et al., 1993; Gaudin et al., 1995; Gaudin et al., 1996). The pH dependent conformational changes are important for RNP delivery into the cell cytoplasm (see § I. 1. 3. b.). The region of G that is crucial for fusion seems to be situated between amino acid 103 and 179 (Durrer et al., 1995). Mutations at the amino acids 124, 127 or 133 lead to altered fusion activities but the viral particles remain still infectious (Fredericksen and Whitt, 1996). Cell fusion and formation of syncytia have also been observed under certain

conditions in infected or transfected cells: for example G transfected neuroblastoma cells but not transfected BHK cells can form syncytia, indicating that some cellular factor is needed for interaction (Morimoto et al., 1992). Transfected HeLa cells also show fusion activity at low pH (Whitt et al., 1991).

Synthesis and folding

Many details about rabies G synthesis and folding are not yet known, and one often extrapolates from vesicular stomatitis virus (VSV) G protein, that shows some similarities (Rose et al., 1982).

Viral glycoproteins share several functional and structural features with other members of the group of integral membrane proteins (for review see Doms et al., 1993): they are important for receptor binding, for membrane fusion and penetration, for the viral morphogenesis at the budding site and for stimulation of virus neutralizing antibody (VNA) production. The majority of these properties are mediated by the ectodomain.

Glycoproteins are translated by the ribosomes of the rough endoplasmatic reticulum (RER) and inserted cotranslationally in the ER in an unfolded form (figure I. 5., p. 14). During folding, the different parts of the proteins are exposed to different conditions: 1) the ectodomain is situated in the ER lumen and processed like membrane or secretory proteins; 2) the transmembrane domain is integrated in the ER membrane, most likely in the form of an α-helix; 3) the cytoplasmic domain is located in the cytosol and therefore processed like cytoplasmic proteins. In the ER, glycoproteins are glycosylated cotranslationally by addition of CA-type (complex-type, high mannose content) carbohydrates composed of mannose, galactose, fucose, N-acetylglucosamine and N-acetylneuraminic acid (sialic acid). Glycosylation is a multi-step process that involves addition and removal of sugar residues (Bergmann et al., 1981). N-linked glycosylation is crucial for appropriate intracellular transport of glycoproteins (Burger et al., 1991; Wojczyk et al., 1995) and the addition of the hydrophilic oligosaccharide core renders folding intermediates more hydrophilic and less likely to form irreversible aggregates.

The ER is the cellular compartment that assures folding and oxidation of newly synthesized proteins. It resembles the extracellular space, but shows a very high Ca^{++} concentration (Baumann et al., 1991), often important for protein folding, as well as a high oxidation potential for the formation of disulfide bonds (Hwang et al., 1992). Further important features of the ER are the presence of "chaperons" and folding enzymes.

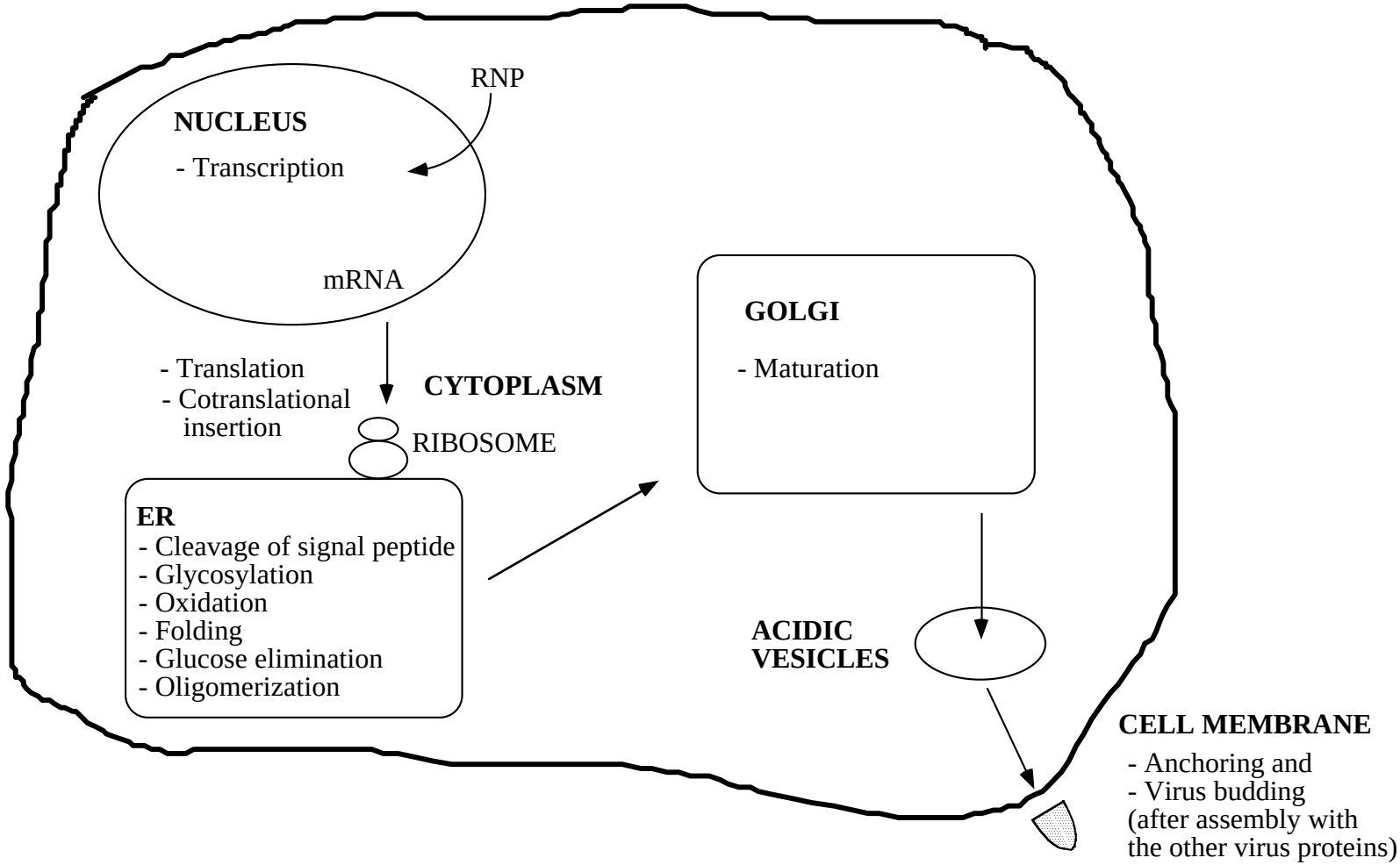


Figure I. 5.: Proposed scheme for rabies glycoprotein syntesis.

Molecular chaperons are ubiquitous proteins that assist polypeptide folding and assembly by binding of nascent polypeptides and therefore prevent both aggregation and release of incorrect or incomplete folded proteins (Ellis and Hemmingsen, 1989). After glycosylation the rabies G protein associates with two chaperons: calnexin and GRP78-BiP (BiP) (Gaudin, 1997). At this stage, some epitopes on the G protein are already detectable. Conformational epitopes (indicating a correct three-dimensional structure) of G are present only after full oxidation of the protein. After oligomerization (trimer formation) the protein migrates to the Golgi apparatus (Bergmann et al., 1981), where it acquires resistance to certain enzymes, such as endo-β-N-acetylclucosaminidase H (Perrin and Atanasiu, 1980), as a sign of full maturation. Finally, G is transported within acidic vesicles to the cytoplasmic membrane (presumably in the fusion-inactive form; Gaudin et al., 1995) and assembled (possibly in interaction with M) with the other viral proteins at the site of budding (see I. 2. 5. c). Altogether, the folding of rabies G shows many similarities with VSV G, even though the folding of rabies G is slower with a half time of 20 min compared to less than 5 min with VSV G.

In addition to synthesis of a full length G rabies infected cells secrete a soluble form of G which lacks the 58 carboxyterminal amino acids. Even though the ectodomain is complete, the soluble G does not induce virus neutralizing antibodies (Dietzschold et al., 1983a), indicating incorrect folding.

POLYMERASE (L)

The RNA-dependent RNA polymerase L (for "large") is the largest (2142 amino acids, 244 kDa) and the less present (25 molecules per virus) rabies virus protein. It is an enzymatic complex showing various activities: RNA dependent polymerase, guanylyl transferase and poly(A) synthetase.

I. 1. 6. Immunity against rabies and rabies vaccines

I. 1. 6. a. Natural immune responses

Numerous mechanisms participate in the immune defense of the organism against rabies (figure I. 6., p. 16). The first obstacle to be met by an infecting rabies virus are macrophages, interferons (IFN α, β and γ) and natural killer cells (NK).

In rabies infection, macrophages have two important roles: they can eliminate the virus by phagocytosis and they process the antigen to induce specific immune response (Turner and Ballard, 1976b; Koprowski et al., 1972; Unanue, 1993).

The first stages of viral infections generally include both the production of IFN (α and β) and the activation of NK cells (Biron, 1994; Welsh et al., 1996). IFNα and β have three major functions: 1) they inhibit viral replication; 2) they

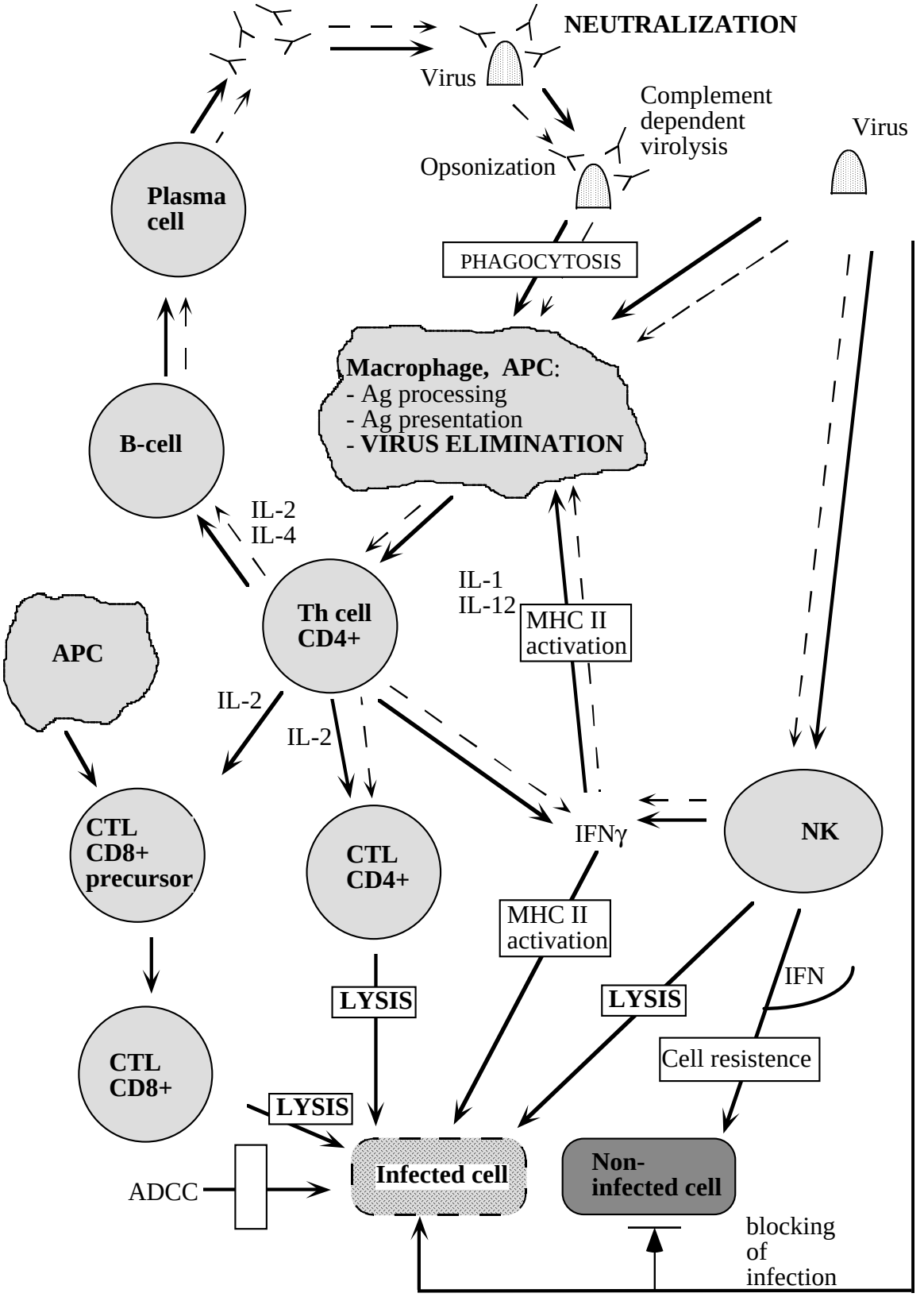


Figure I. 6. Scheme of the immune response after (modified after Süss and Sinnecker, 1989).

infection

immunization

induce the expression of the major histocompatibility complex (MHC) I by most cells and 3) they activate NK cells that are capable to kill virus-infected cells. Concerning IFNγ, its most important function is the activation of macrophages that in turn express MHC I and II molecules. Moreover IFNγ has a direct antiviral effect (Baer et al., 1988) even though to a lesser extent than IFNα and β (Roitt et al., 1993; Janeway and Travers, 1996).

After experimental rabies vaccination NK cells, activated Th1 cells and cytotoxic T lymphocytes (CTL) produce IFNγ (Celis et al., 1986). The protective effect of IFNs has clearly been demonstrated since mice have been protected against rabies after IFN or IFN-inducer injection (Wiktor et al., 1972; Baer et al., 1988; Lin et al., 1993). Protective IFN levels may even be induced by a non-rabies vaccine, provided this vaccine is administered at the moment of the challenge (Baer et al., 1988; Lodmell et al., 1989). Exogenous IFN administered at the time of rabies vaccination increases the immune response against rabies significantly (Mifune et al., 1980; Schijns et al., 1994). After rabies vaccination, IFN is produced much earlier than VNA and might therefore be more important in the early stage of infection (Mifune et al., 1980).

The role of IFNγ has been discussed for the protective effect of RNP against a peripheral challenge (see § I. 1. 7. b.) which can be overcome by injection of anti-IFNγ antibodies, leading to the hypothesis, that IFNγ could block the virus spread into the CNS (Dietzschold et al., 1989).

NK cells are induced after rabies immunization (Perrin et al., 1988) and after rabies infection (Bahloul et al., 1997) but their importance in anti-rabies immunity is not yet clearly established (Hemachudha, 1994).

I. 1. 6. b. Specific immune response

After their capture by macrophages and other antigen presenting cells (APC: dendritic cells and B lymphocytes) rabies antigens are presented to specific $CD4^+$ or $CD8^+$ cells. Their stimulation induces the production of cytokines, such as interleukin-2 (IL-2), IL-4 and IFNγ. $CD4^+$ cells can provide help for specific B cells that produce VNA (for review see Roitt et al., 1993; Janeway and Travers, 1996).

T cell response

T lymphocytes can be divided into two different subsets: $CD4^+$ that interact with MHC II of APC and $CD8^+$ that interact with MHC I which can be found on almost all cell populations. The function of the $CD4^+$ subset consists mainly in T helper cell function whereas $CD8^+$ cells are mostly cytotoxic T cells (Bierer et al 1989). The importance of T cells for anti-rabies immunity has been demonstratedin nude mice, which, in contrast to their normal litter mates were

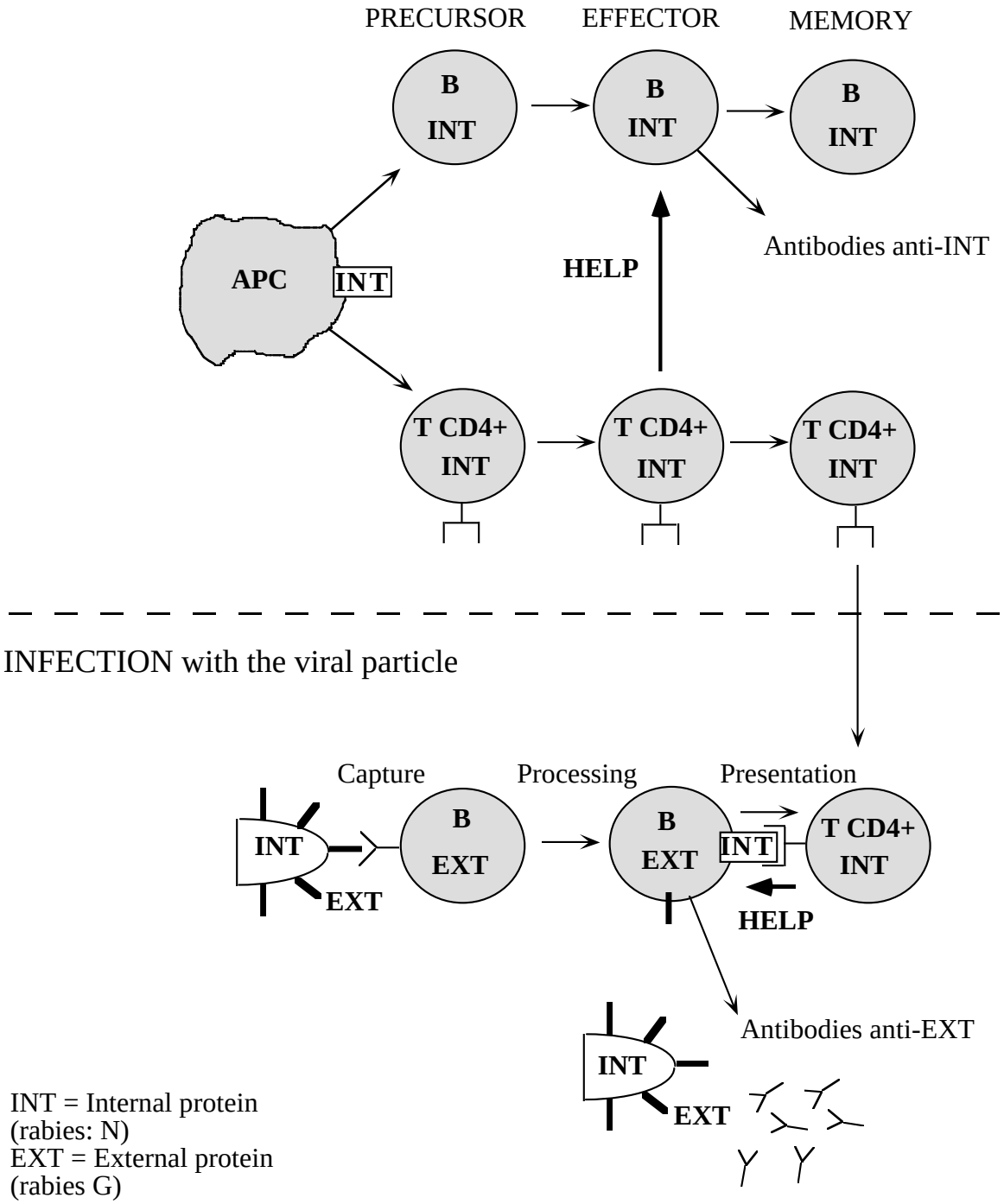


Figure I. 7.: Proposed scheme for the cross-help mechanism (after J. Virelezier).

unable to mount an antibody response after rabies vaccination and to resist a challenge of infectious virus (Turner, 1976a; Mifune et al., 1981).

Following the type of antigen concerned, different patterns of cytokines are secreted which in turn stimulate T helper cells (Th) to produce different cytokines. IL-12 and IFNγ will cause a lymphocyte cytokine secretion of the Th1 type (IFNγ, IL-2 and lymphotoxin production) with a B cell help that induces mainly IgG2a isotype production. IL-4 causes rather a Th2 type response (IL-4, IL-5 and probably IL-6 production) which leads to IgE production by stimulated B cells (Mosmann and Coffman, 1989).

The Th response in the case of immunization with inactivated rabies virus is of the Th1 type with an important production of IL-2 and IFNγ after stimulation (Celis et al., 1986; Perrin et al., 1988; Ertl et al., 1989b) and secretion of the IgG2a subclass of antibodies, as it has been described to be typical for viral infections in mice (Couterlier et al., 1988). The importance of Th cells has been established by depletion experiments: a depletion of $CD4^+$ cells or use of $CD4^+$ knockout mice leads to a decrease in both, VNA production and protection (Perry and Lodmell, 1991; Xiang et al., 1995b). However, $CD4^+$ cells do not act independently in protection since depleted animals can be protected if immune serum is administered (Celis et al., 1990).

The help of B cells by Th cells provided in the case of rabies leads to a switch from the IgM to the IgG2a isotype. A particular mechanism of T help has been discussed in the case of the priming by the N protein (see § I. 1. 7. b.; Dietzschold et al., 1987b; Fu et al., 1991): the "intrastructural help" or "cross-help" as described with influenza virus (Russell and Liew, 1979). In that case, memory T cells recognizing some internal component of the virion are capable of cooperation with B cells specific for a surface protein (and which are capable of antigen presentation and antibody production) and thus stimulate the production of antibodies directed against the surface protein. A possible mechanism for this phenomenon is described in figure I. 7. Actually, the Th response against rabies has been shown to display cross-reactivity between the different sero-genotypes (Dietzschold et al., 1987b; Celis et al., 1988a; Perrin et al., 1996b).

Beside IFNγ (see § I. 1. 6. a.), interleukin-2 (IL-2) plays a pivotal role in the anti-rabies immune response. Indeed, exogenous IL-2 has been shown to amplify protection induced by anti-rabies vaccines (Perrin et al., 1988; Nunberg et al., 1989) and it is able to activate CTL, Th, B cells and NK (Perrin et al., 1989). The production of IL-2 upon restimulation has been reported to be a good indicator for anti-rabies protection (Wiktor et al., 1977; Joffret et al., 1991). It is important to note that in natural rabies infection the beneficial function of specific Th cells is suppressed (Perrin et al., 1996b).

The production of CTL after rabies vaccination with an attenuated virus is important (Wiktor et al., 1977), but CTL alone cannot protect against a challenge. Animals that are depleted of $CD8^+$ cells but still have VNA are protected (Perry and Lodmell, 1991) and animals that have specific CTL but no VNA are not (Celis et al., 1990).

The role of $CD4^+$ and $CD8^+$ cells in rabies is not always positive since it has been suggested that they are responsible for the paralytic form of rabies due to the nerve destruction by these cytolytic cells (Weiland et al., 1992).

B cell response and antibodies

In rabies immunity, VNA are the crucial element of protection (Cox et al., 1977; Bunschoten et al., 1989; Lafon et al., 1983; Wunderli et al., 1991). Different mechanisms of rabies virus neutralization by antibodies have been suggested: 1) neutralization of extracellular virus, and 2) mediation of both complement and antibody-dependent cellular cytotoxicity (ADCC) of infected cells (Pereira at al., 1982; Davis and Metzger, 1983). Protective antibodies are of the IgG subtype while the IgM subtype does not display any protective activity (Turner, 1978; Mifune et al., 1980). VNA have been shown to inhibit *in vitro* the cell-to-cell spread of rabies virus, but more efficiently in fibroblastic and epitheloid cell than in neuroblastoma cells. They might therefore be of particular importance at the inoculation site (Lodmell and Ewalt, 1987). Post-exposure vaccination of humans is thus often accompanied by rabies immunoglobulin injection at the bite site (see § I. 2. 1. b.).

In rare cases VNA have been responsible for enhanced death by rabies, the "early death" phenomenon: insufficiently immunized animals succumbed to virus challenge earlier than the non-immunized litter mates (Andral and Blancou, 1981; Prabhakar and Nathanson, 1981). The formation of immune complexes might be one explanation for this effect (Schumacher et al., 1992).

Non-neutralizing antibodies (nVNA) have been discussed as one of the mechanisms of protection achieved by N (see § I. 1. 7. b.).

I. 1. 6. c. Relative importance of the different immune responses against rabies (figure I. 6.)

Among the different elements of the immune response against rabies, one can clearly distinguish some that are more involved in the protection achieved than others. After preventive vaccination the most important element of the immune response are VNA. A person is considered to be protected against rabies when a VNA titre of $\geq$ 0.5 international units (IU) per ml serum has been measured. After post-exposure vaccination only the protective role of VNA, Th cells and IFN has been demonstrated *in vivo*. CTL are produced but they do not confer protection.

I. 1. 7. Role of viral proteins in the protection against rabies

The most important viral proteins involved in the protection against rabies are G and N. Antigenic variation of G between the different genotypes is very important (Wiktor and Koprowski, 1978; Flamand et al., 1980), whereas N exhibits less variability: isolates that belong to different genotypes present up to 92% aminoacid homology on the N protein (Kissi et al., 1995).

I. 1. 7. a. Glycoprotein

The antigenic sites of G of the ERA strain have been identified using monoclonal antibodies (figure I. 8. a., p. 22) (Flamand et al., 1980; Lafon et al., 1984). Two immunodomimant sites are recognized by the majority of antibodies: site II and site III. Site II is a conformational epitope, consisting of two parts, IIa (aa 198-200) and IIb (aa 34-42) (Prehaud et al., 1988), that are joined by disulfide bonds (Dietzschold et al., 1982). Under denaturing conditions this site is therefore not recognized by the corresponding antibody (Libeau et al., 1984; Lafon et al., 1985a). Site III, too, is a conformational site, represented by at least three epitopes that consist of amino acids in close relation to each other (aa 330-338). It includes a forth more distantly located epitope (aa 357)(Seif et al., 1985; Wunner et al., 1988). Three minor sites have also been identified: a (aa 342-343), b and c (Prehaud et al., 1988; Benmansour et al., 1991). Site b and c contain the former site VI (aa 264 Dietzschold et al., 1983b; Bunschoten et al., 1989; Benmansour et al., 1991). Site I and site IV (Lafon et al., 1983) have only been identified by one monoclonal antibody each and are therefore not retained in this schema. The relative importance of the different epitopes has been estimated by the number of neutralizing monoclonal antibodies directed against them that have been isolated (fig. I. 8. b. Benmansour et al., 1991).

G is the only rabies protein able to induce VNA (Wiktor et al., 1973; Cox et al., 1977) and can achieve without the presence of other antigenic structures total protection against an intracerebral challenge (Atanasiu et al., 1974; Cox et al., 1977; Perrin et al., 1985c; Takita-Sonada et al., 1993). G specific monoclonal antibodies that show virus neutralizing activity *in vitro* protect against a peripheral challenge *in vivo* (Dietzschold et al., 1990a).

The three-dimensional structure of G and its environment are highly important for the induction of VNA (Perrin et al., 1988). Purified G forms aggregates ("rosettes") that induce much less VNA than correctly folded G, indicating that G needs to be anchored on a membrane (viral particle or liposomes) (Cox et al., 1980; Perrin et al., 1984; Perrin et al., 1985b). Similarly, the soluble G form that lacks the transmembrane region induces 15 times less VNA than the native G (Dietzschold et al., 1983a).

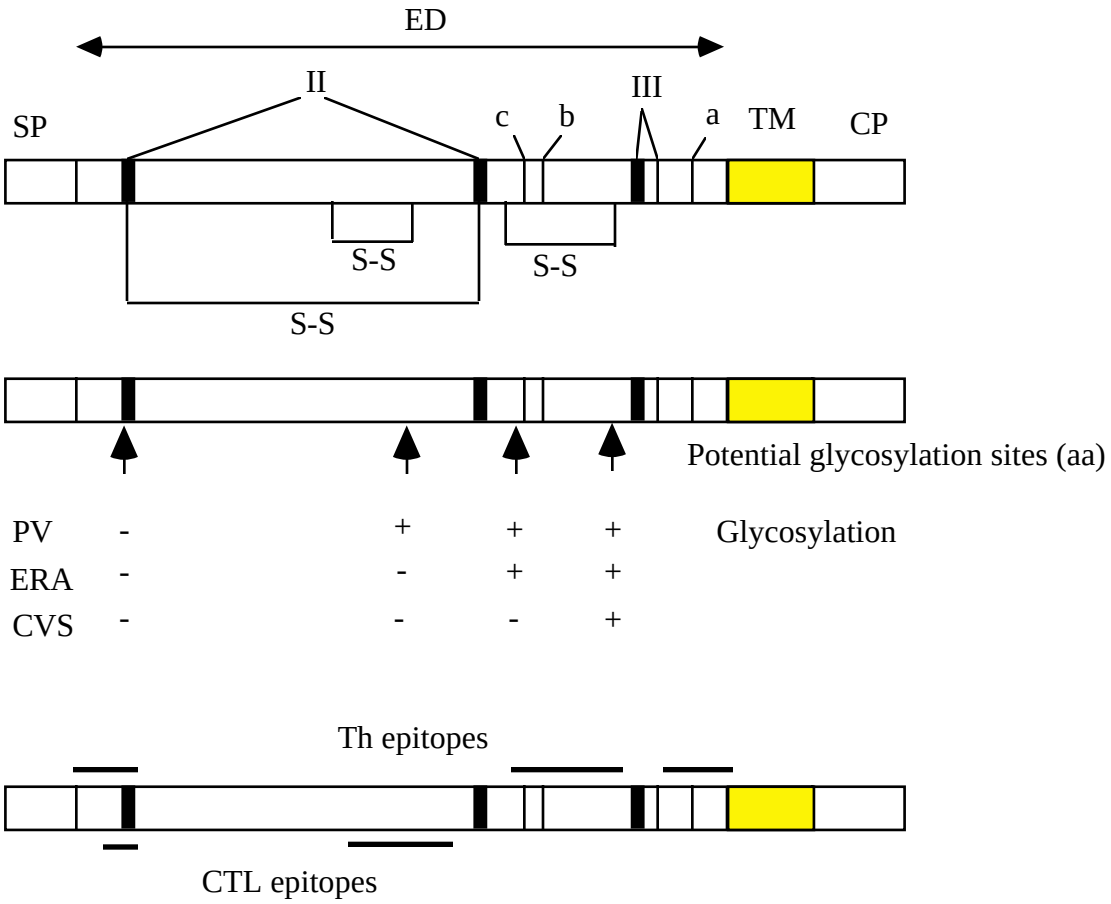


Figure I. 8. a.: Rabies G structure and antigenic sites
(SP = Signal peptide; ED = Ectodomain; TM =Transmembrane domain; CP = Cytoplasmic domain).

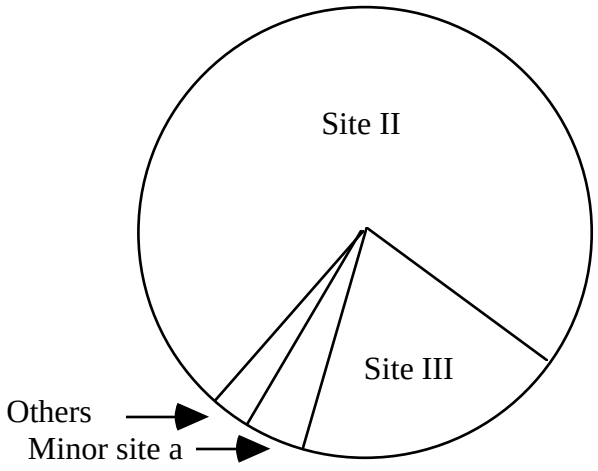


Figure I. 8. b.: Relative frequency of VNA isolation against the antigenic sites of rabies G (Benmansour et al., 1991).

The G protein is recognized both by Th cells (MacFarlan et al., 1984; Celis et al., 1988b; Xiang et al., 1995b; Bahloul et al., 1997) and CTL (Wiktor et al., 1984; Celis et al., 1988b). Whereas infectious virus, inactivated virus or subunit G associations induce a Th response, only infectious viral particles or "naked" DNA coding for G can induce CTLs. Three sequential synthetic peptides deduced from the aminoacid sequence of rabies G have been shown to stimulate the Th cell response: aa 1-44, aa 244-291 + 386-452 and aa 292-323 (MacFarlan et al., 1984). Aa 18-44 are involved in both, Th and CTL responses (MacFarlan et al., 1986). Another CTL epitope is located between aa 130 and aa 178, containing a single disulfide loop (159-169) (Wunner et al., 1985; MacFarlan et al., 1986). Concerning the recognition of G by Th cells, its presentation seems to be of similar importance as for the presentation to B cells (see § I. 1. 6. b.). Anchored on liposomes, purified G induces as much IL-2 production upon *in vitro* stimulation of lymphocytes as inactivated virus (Oth et al., 1987; Perrin et al., 1988).

I. 1. 7. b. Nucleoprotein

Antigenic structure

Three antigenic sites have been described on the N protein of the ERA strain (Lafon and Wiktor, 1985b). Site I (aa 374-383) and site III (aa 313-337) contain continuous epitopes whereas site II is discontinuous (Lafon and Wiktor, 1985b; Dietzschold et al., 1987a). The N protein is also an efficacious Th cell inducer and a Th epitope has been identified (aa 404-418) (Ertl et al., 1991).

Protective properties and subunit vaccine

Viral RNP and N protein have been shown to induce partial protection against intramuscular challenge whereas no protection is observed when the challenge virus is injected intracerebrally. However, large amounts (for example 10 µg of RNP or N) accompanied by Complete Freund's Adjuvant (CFA) must be injected. Similar results have been obtained with recombinant N proteins: purified N protein from recombinant baculovirus (N-Bac) (Fu et al., 1991), or 10^6-10^8 plaque forming units (PFU) of recombinant poxvirus (N-Vacc) (Lodmell et al., 1991; Sumner et al., 1991; Fekadu et al., 1992) induced partial protection against a homologous intramuscular challenge. Furthermore, cross-protection after nucleoprotein injection has been reported: RNP from Mokola and ERA virus can protect against CVS, and Duvenhage challenge respectively (Dietzschold et al., 1987b).

N has often been discussed to be important component in a subunit vaccine against rabies because of its capacity to induce Th cells and its conservation between the serotypes. N might therefore have a possible rule in a vaccine with an broadened spectrum (Dietzschold et al., 1988). However, the RNP complex could not be a part of a subunit vaccine: it has to be extracted from virus particles or infected cells which

is expensive and carries the risk of a contamination with viral RNA (Schneider et al., 1973). Consequently, recombinant N protein is to be preferred even though it elicits often less high immune responses than RNP, possibly because of the greater stability of the protein in the RNP complex or the difference of antigen processing in the case of an N-recombinant virus (Fu et al., 1994). On the other hand, it has been discussed whether N could be used as priming for a better efficacy of vaccination or for faster virus clearance after subsequent infection. Whereas RNP (Tollis et al., 1991) and N-Bac (Fu et al., 1991) have been reported to prime for rabies vaccination or for the immune response after rabies infection, no priming has been observed with N-Vacc (Sumner et al., 1991) or with peptides carrying epitopes of N (Ertl, 1989a).

After oral administration N protein is able to induce T-cell response and to prime for a following vaccination. However, coadministration of N-Vacc did not improve the protection achieved by G recombinant vaccinia virus (G-Vacc) in oral vaccination of racoons (Sumner et al., 1991). Even though N is considered to be a superantigen (see below) there is no tolerance after an oral immunization, possibly because N is a rather weak superantigen (Hooper et al., 1994).

Combinations of G and N have been tested for their performance in a subunit vaccine: liposomes containing both G and RNP only induced a slightly higher protection against intracerebral challenge than G liposome alone (Dietzschold et al., 1987b). A tandem peptide carrying a Th epitope of N and a B cell epitope of G protected against intramuscular challenge (Dietzschold et al., 1990b). On the other hand G-Vacc and N-Vacc injected at different sites did not raise higher titers of VNA than G-Vacc alone (Fekadu et al., 1992).

Thus it can be concluded that while the importance of G in the immune response against rabies virus infection is clearly established, the role of N is still object of controversy. There are several arguments that N might be an important factor in a subunit vaccine, but on the other hand it has not yet been determined whether N would really be able to improve the immunity achieved by G concerning amplitude or the spectrum of protection.

Immunological properties of N

The mechanism of the protection induced by the N protein is not yet fully understood. In the above described experiments that show a protection by N, no VNA have been detected. The protection must therefore have been achieved by other means, such as the Th response (Dietzschold et al., 1987b; Dietzschold et al., 1989), anti-N-antibodies and/or IFNγ production (Fujii et al., 1994; Tollis et al., 1991). Indeed, RNP is able to induce specific Th-cells (Celis et al., 1986) but for stimulation of IL-2 production *in vitro* RNP is less potent than the G anchored onto liposomes (Perrin et al., 1988). N also induces non-neutralizing antibodies that seem to be important for protection: higher anti-RNP antibody titres are correlated with

higher survivor rates in RNP or N immunized mice (Fu et al., 1991). Furthermore, anti-N serum is able to protect under certain conditions against a weak virus challenge (Lodmell et al., 1993). Anti-N antibodies might act directly on the virus replication since anti-N or anti-P antibodies have been reported to inhibit virus replication *in vitro* (Lafon and Lafage, 1987). In addition, anti-N antibodies seem to improve indirectly the immune response against N: administration of plasma from vaccinated animals augments the Th response against virus, G and N (virus>N>G). This might be a mechanism involved in the post-exposure protection (Celis et al., 1985). Protective non-neutralizing antibodies have as well been reported in the case of other viruses, for example VSV (Lefrancois, 1984).

The role of IFN in protection achieved by N has been established since mice that are protected after immunization with N raise high levels of IFN whereas mice that have been previously depleted in IFN (injection of anti-IFN antibodies) are not protected (Dietzschold et al., 1989).

Superantigen properties

In contrast to ordinary antigens, superantigens highly stimulate T cells without binding to the peptide-binding groove of the MHC molecule, but instead to the upper surface of both the MHC class II molecule and the Vβ region of the T-cell receptor. This interaction takes place without former processing of the antigen. As one superantigen can bind to different fragments of the Vβ chain it can stimulate 2-20% of all T cells - the polyclonal response following this phenomenon inhibits a normal immune response (Kappler, 1987; Dellabona P., 1990; Janeway, 1991). The presence of superantigens has been reported in bacteria, *Staphylococcus aureus* and *Mycoplasma* (Marrack and Kappler, 1990; Cole and Atkins, 1991), as well as in infections by exogenous and endogenous mouse mammary tumor retroviruses (Acha-Orbea, 1992; Acha-Orbea et al., 1993; Huber, 1992).

Several arguments exist to include N in the family of superantigens: both N and RNP are able to bind directly on MHC II without any processing (Lafon et al., 1992; Lafon, 1993) inducing the proliferation of $V\beta6^+$ cells which are the target cells for superantigens (Lafon, 1993). This property might be responsible for the paralytic form of rabies as the immune pathology observed in rabid mice depends on the presence of the Vβ receptor (Lafon M., 1996). Two possible mechanisms for this phenomenon are proposed: 1) induction of IFNγ, which afterwards induces the formation of MHC I - receptors on neurons and thus renders them sensitive for an attack by CTLs; 2) induction of antibodies triggering an antibody-dependent-cell-cytotoxicity (ADCC) by killer microglia (Lafon et al., 1994).

1. 1. 7. c. Other rabies virus proteins

Concerning the phosphoprotein (P), two antigenic sites have been described (Lafon and Wiktor, 1985b; Larson et al., 1991). P is also able to induce a strong CTL response, but stimulates Th and B cells only poorly. It induces no (Larson et al., 1992) or only partial protection against intramuscular challenge (Takita-Sonada et al., 1993).Very few data are reported on the immunological properties of the matrix protein (M): it induces CTLs (Cho et al., 1987) and low antibody levels but it is unable to confer protection (Takita-Sonada et al., 1993).

I. 2. Rabies vaccines and protection against lyssaviruses

I. 2. 1. Classical vaccines

Since the first rabies vaccine that has been developed by Louis Pasteur (Pasteur, 1885), consisting in subcutaneous inoculation of spinal cord suspension derived from rabid rabbits, different generations of vaccines have been developed (table I. 9.; for review see Wiktor et al., 1988; Celis et al., 1989; Perrin et al., 1990; Plotkin, 1993).

I. 2. 1. a. Different generations of vaccines

The first generation of rabies vaccines consisted in phenol or heat inactivated viruses produced on nervous tissue. In spite of often severe side effects that contribute to the burden of rabies on public health, those vaccines are still used in developing countries since they are easy to prepare and less expensive than the more recent generations of vaccines (Meslin et al., 1994). In order to eliminate these side effects, the development continued: first towards myelin-free vaccines prepared from neonatal mouse brains, then to cell culture vaccines, free of neuronal tissue.

In Europe, for example, the following types of vaccines are produced today: purified duck embryo cell vaccine (PDEV), dog kidney cell vaccine, purified chicken embryo cell vaccine (PCEC) and vero cell vaccine (Sureau, 1992). Within the veterinary field there is an additional type of vaccination available: the oral vaccination of wildlife (for review see Winkler and Bögel, 1992). The first vaccination campaigns were carried out using live modified virus vaccines, derived mainly from the SAD-ERA strain. In some countries these vaccines were replaced by recombinant vaccinia virus expressing rabies glycoprotein (G-Vacc, see § I. 2. 3. a.) (Brochier et al., 1990). For safety risks with vaccinia virus in humans, SAD strains are still used for oral vaccination, even though they have been shown to maintain a certain pathogenicity against wild rodents (Artois et al., 1992). The oral

GENERATION	SOURCE OF PREPARATION	IMMUNO-GENICITY	HUMAN/ VETERINARY (H/V)
1. Whole inactivated virus	**NERVOUS TISSUE**		
	- adult animals (sheep, goat, rabbit)	very poor	H
	- new-born mice	poor	H
2. Whole inactivated virus	**PRIMARY CELL EXPLANTS**		
	- embryos (chicken, duck)		
	- kidney (hamster, dog, pig)	good	H
	- kidney (bovine fetus)	good	H
		very good	H
3. Whole inactivated virus	**DIPLOID CELLS**		
	- human (Wi-38, MRC-5)	good	H
	- rhesus monkey (fibroblasts)	good	H
	HETEROPLOID CELLS		
	- Vero	very good	H
	- BHK-21	good	V
4. Avirulent active virus	Mutation aa 333	very good	V
5. Recombinant active virus	G-recombinant Vaccinia	very good	V

Table I. 9.: Vaccines against rabies used today.

vaccination of wildlife, together with eradication of rabies in domestic dogs has lead to a significant reduction of rabies incidence in Europe.

I. 2. 1. b. Anti-rabies vaccination of humans

Whereas the veterinary use of rabies vaccines is limited to preventive measures, vaccination of humans takes place mainly after exposure to a rabid animal. Different regimens of vaccination are recommended (WHO, 1996), varying in number and site (muscle or skin) of injections. In the case of severe exposure vaccination is often accompanied by injection of human rabies immunoglobulin (HRIG). However, for financial reasons HRIG is often replaced by equine immunoglobulins (ERIG). In the future, monoclonal human antibodies might be an alternative (Schumacher C. L., 1989). Serum can only be used in addition to vaccination, on its own, it does not protect against rabies (Sikes et al., 1971). A major draw back of immune serum is that it inhibits VNA production after vaccination (Baer et al., 1988). This effect could be partially overcome, if the number of vaccinations was increased or if the vaccination took place at least 15 days after the immunoglobulin treatment or if serum was administered 12-24 h after vaccination (Wiktor et al., 1971). Patients who had previously received a complete pre- or post-exposure course or who have been shown at some time in the past to have rabies neutralizing antibodies (>0.5 IU/ml) only need an abbreviated injection schedule without immunoglobuline treatment.

I. 2. 1. c. Protection against the lyssaviruses

With classical rabies vaccines, cross-protection has been correlated to genetic and antigenic distance between the vaccinal strain and the challenge strain (Koprowski et al., 1985). The vaccines available today are produced with "fixed strains" (Pasteur virus, PV; Pitman Moore, PM; Challenge virus standard, CVS) (Sureau, 1988). All of these vaccinal strains belong to genotype 1 (fig. I. 10.). They show a different degree of cross-protection towards other genotypes: 1) EBL2 (genotype 6) seems to be immunologically close and is very well protected by a vaccine of genotype 1 (Bahloul et al., 1997); 3) the African viruses are very distant and are not covered by classical vaccine in the case of the genotypes 2 and 3, and only to a small extend in the case of genotype 4 (Fekadu et al., 1988): 4) the cross-reactivity with EBL1 (genotype 5) varies according to the vaccine virus strain: the PM virus strain confers protection against EBL 1 only in some cases (Lafon et al., 1986; Fekadu et al., 1988), whereas PV protects better (Lafon et al., 1988) and CVS induces a titer of neutralizing antibodies against EBL1 that corresponds to 25% of the titer against CVS (Herzog et al., 1991). This intermediate position of EBL1 is confirmed by its molecular characterization: EBL1 is more closely related to genotype 4 than to genotype 1 and to genotype 6 (Bourhy et al., 1992).

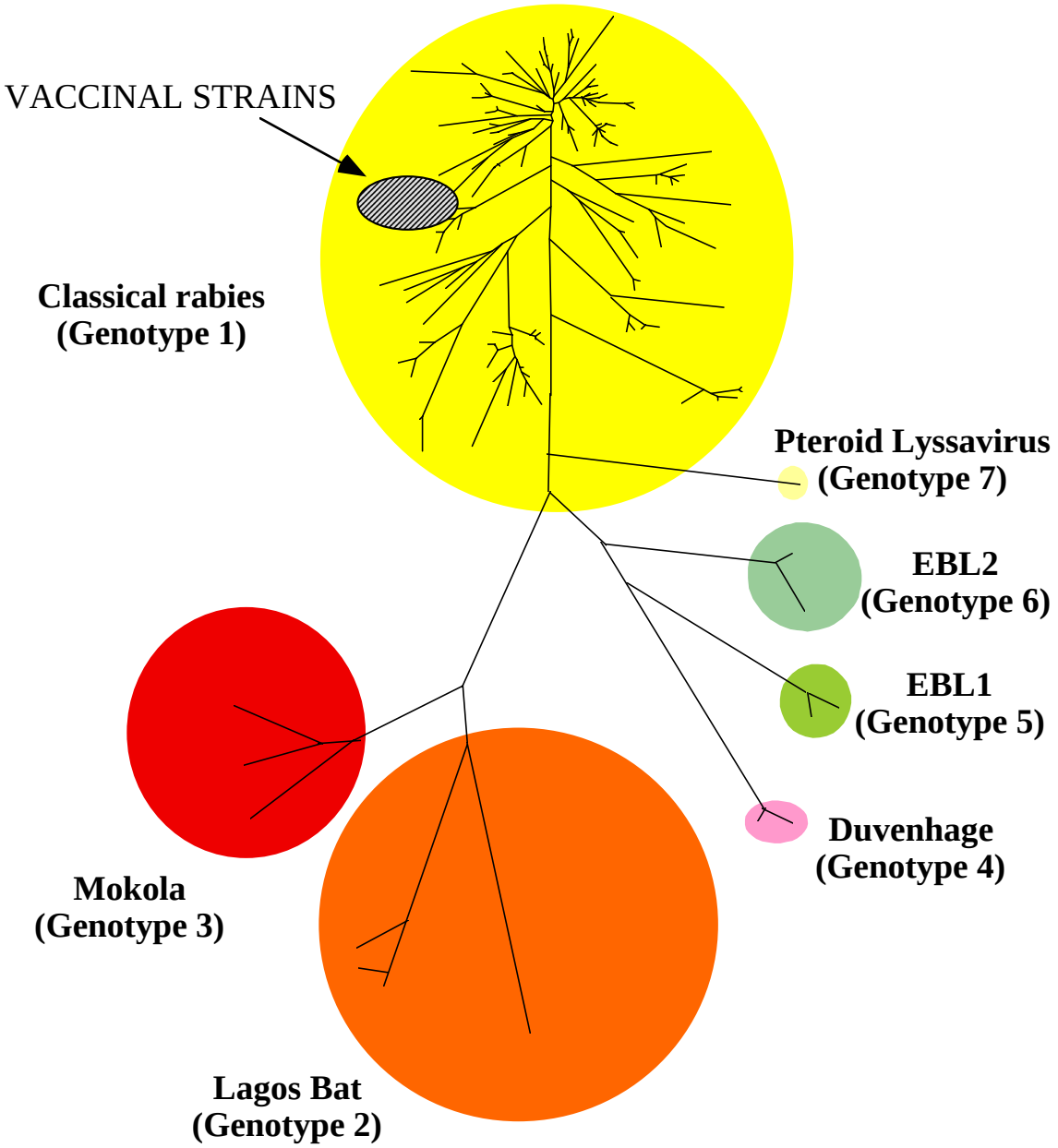


Figure I. 10.: Position of the rabies vaccinal strains within the phylogenetic tree of the lyssaviruses.

I. 2. 2. Synthetic peptide vaccines

Synthetic peptide vaccines have several advantages to traditional vaccines: they are safe, they induce well-defined monofunctional immune responses and they can be produced with high reproducibility and exquisite purity in large quantities. Nevertheless, their immunogenicity is low and the monospecificity of the immune response allows mutating pathogens to escape from immunosurveillance (Ertl and Xiang, 1996a). Several approaches of synthetic peptide vaccine against rabies have been tested: 1) a peptide carrying an immunodominant T cell determinant of the N; 2) a peptide containing a B cell epitope of N; 3) a tandem peptide containing both previously mentioned N epitopes ; and 4) a tandem peptide containing a Th cell epitope of N and a B cell epitope of the G protein. Both tandem peptides injected together with adjuvant were able to induce protection against a peripheral challenge with CVS virus (Dietzschold et al., 1990b).

I. 2. 3. Recombinant vaccines

Recombinant vaccines against rabies are based either on the injection of recombinant viruses or of recombinant proteins produced *in vitro*. Recently, rabies glycoprotein has even been expressed in transgenic plants (McGarvey et al., 1995).

I. 2. 3. a. Recombinant viruses

Apart from recombinant vaccinia viruses expressing rabies virus G that are used for wildlife immunization, recombinant rabies vaccines have so far only experimental importance. Recombinant vaccinia viruses (poxviruses) have been successfully used for expression of foreign antigens such as hepatitis B virus surface antigen (Smith G. L. et al., 1983a), herpes simplex virus glycoprotein D (Paoletti et al., 1984) and influenza hemagglutinin (Smith G. L. et al., 1983b). Concerning rabies, all structural proteins have been expressed in vaccinia viruses (Takita-Sonada et al., 1993). Particular stress has been laid upon G (G-Vacc) (Kieny et al., 1984; Wiktor et al., 1984; Fujii et al., 1994) and N (N-Vacc) (Sumner et al., 1991; Fekadu et al., 1992) and both have shown the same immunological features as the corresponding viral protein. G-Vacc has been used with much success for wildlife immunization in Europe (see § 1. 2. 1.).

Other poxviruses have been used for the expression of rabies antigens: racoon poxvirus recombinants expressing N (Lodmell et al., 1991) and canarypox recombinants expressing G (Cadoz et al., 1992; Taylor et al., 1995). The canarypox vector has the considerable advantage not to be replicative in human cells. It would therefore be more suitable than vaccinia virus that has been forbidden for human use because of safety risks. The same advantage as with recombinant canarypox is valid for recombinant adenovirus that expresses rabies G (Prevec et al., 1990).

I. 2. 3. b. Recombinant proteins and the baculovirus expression system

Rabies G that was produced in Escherichia coli was not immunogenic (Yelverton E., 1983; Lathe et al., 1984) whereas G expressed in yeast was able to protect against an intramuscular challenge but not against an intracerebral virus injection (Klepfer S. R., 1993).

A very thoroughly investigated eukaryotic expression system are the baculoviruses (for review see Devauchelle and Cerutti, 1993; Summers and Smith, 1988). These viruses only infect arthropods and even if they are highly virulent for some insects, they are neither pathogen for vertebrates nor for plants. One of the most studied baculoviruses is the *Autographa californica* Nuclear Polyhedrosis Virus (AcNPV) that has been isolated from insect larvae. Like other baculoviruses it has a biphasic life cycle (fig. I. 11. a.) that leads to two different forms of viruses: 1) extracellular virus that buds from the cells is responsible for cell-to-cell infection in cultured cells or in the insect host; 2) virus particles embedded in occlusions formed by a virus encoded protein (named polyhedrin) assure the horizontal transmission of the infection. The occlusions appear 24h after infection and are shed into the environment after the death of the infected insect larvae. They protect the virus until they are taken up by another insect larvae and dissolved by the alkaline intestinal secretions, thus releasing the virus that subsequently enters another infectious cycle.

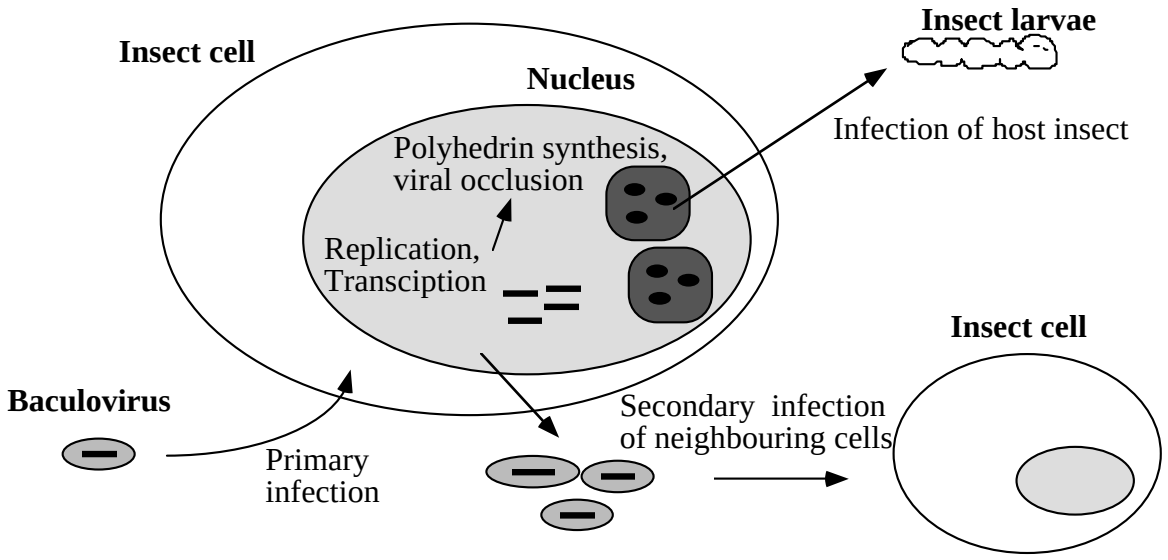


Figure. I. 11. a. Baculovirus life cycle (Summers et al. 1987).

In cell culture, the polyhedrin gene is not essential for the infectious cycle and as polyhedrin is produced to a very high amount in the infected insect cells (up to 1 mg per 1 to $2x10^6$ cells), this gene has been chosen to be replaced by a foreign gene for the expression of recombinant proteins (Smith et al., 1985). The foreign gene is cloned into a plasmid transfer vector. By homologous recombination with the wild type virus a recombinant baculovirus vector is obtained, that will produce its gene product in infected insect cells (fig. I. 11. b.). The baculovirus expression system is widely used for its high yield of protein products (Smith, G. E. et al., 1983; Matsuura et al., 1987). In addition, expressed proteins undergo eukaryote-specific post-translational modifications such as glycosylation and polymerization (Luckow et al., 1988). Moreover, large-scale production of recombinant proteins by baculovirus expression system can be achieved using airlift bioreactors (Maiorella et al., 1988; Murhammer and Goochee, 1988).

Different rabies proteins have been expressed in the baculovirus expression system. The expression is associated with characteristic cellular structures: the expression of G is accompanied by a high level of cytoplasmic vacuolization, the

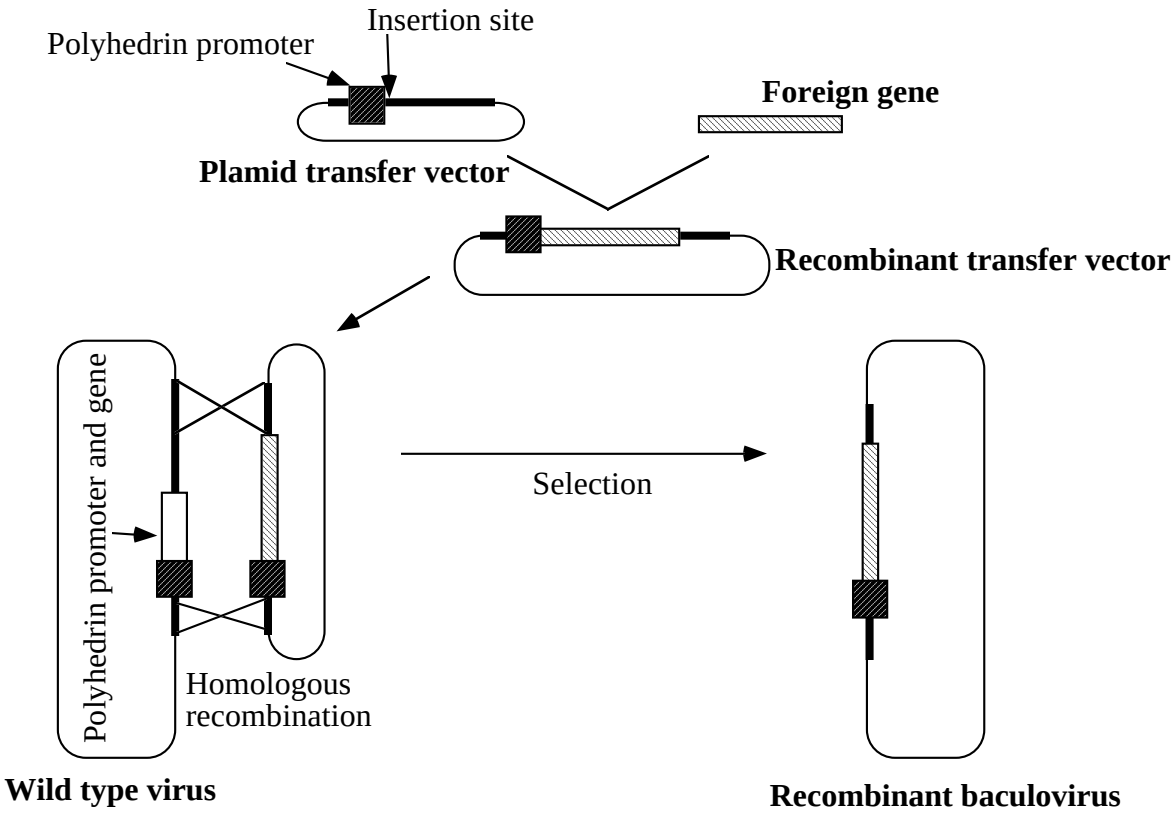


Figure I. 11. b.: Construction of a baculovirus expression vector (Devauchelle et al. 1993).

expression of N by ring-like structures (Pinto et al., 1994). Rabies G protein of the Nishigahara strain (genotype I) produced by baculoviruses (G-Bac) is antigenically similar to wild virus G, even though the molecular weight is slightly lower. At low pH it shows a fusion activity, an indication for pH dependent conformational changes. Only the glycosylation (number and structure of carbohydrate side chains) seems to be different, leading to two glycosylated species of recombinant G protein: both migrated faster than the authentic G protein (Tuchiya et al., 1992). Similar phenomena were also reported in the case of the expression of G protein of the CVS virus (Prehaud et al., 1989) and with VSV glycoprotein (Bailey et al., 1989) or influenza virus hemagglutinin expressed in insect cells (Kuroda et al., 1990). But the biological features of recombinant rabies G are still similar to those of the viral protein, including fusion activity and protective immunity: cells expressing the recombinant G of PV induce protection against genotype 1 (Prehaud et al., 1989) cells expressing the recombinant G of Mokola induce protection against genotype 4 (Tordo et al., 1993). The equivalent functional properties indicate a similar three-dimensional structure (Tuchiya K., 1992). This is crucial, as a correct folding is necessary for expression on the cell surface (Wiktor et al., 1984).

I. 2. 4. Naked DNA

I. 2. 4. a. DNA-based immunization

Initially, the technique of direct gene transfer by inoculation of plasmid vectors was developed for postnatal gene therapy (Williams et al., 1991). After intramuscular injection, the muscle cells were found to readily take up the DNA and to express the foreign gene (Wolff et al., 1990; Wolff et al., 1991; Davis et al., 1993b). Since the expression of such a foreign gene has been shown to induce antibodies (Tang et al., 1992), this model has been tested with different viral (Ulmer et al., 1993; Fynan et al., 1993; Wang et al., 1993; Davis et al., 1994; Manickan et al., 1995), parasite (Sedegah et al., 1994; Xu and Liew, 1994) and bacterial antigens (Anderson et al., 1996). In most cases the results were rather similar, indicating stimulation of antibody production, CTL and, if tested, protection.

Plasmid vectors

The foreign gene is carried by a eukaryotic expression vector that displays certain features. The foreign genes are mainly under control of an eukaryotic promoter, such as the early promoter of cytomegalovirus (CMV) (Davis et al., 1993b) or the simian virus (SV) 40 promoter. In general, the SV 40 promoter leads to much less expression than the CMV promoter (Davis et al., 1994; Xiang et al., 1995c) as confirmed in the case of rabies G (Xiang et al., 1995; Bahloul et al., 1997). The foreign gene is usually cloned into a polylinker behind the promoter. A polyadenylation site situated behind the polylinker assures the stability of the

transcribed mRNA. For amplification of the plasmid in bacteria, it contains a bacterial replication origin and an antibiotic resistance gene for selection. Another characteristic of bacterial plasmid vectors has been demonstrated to be important for the immune response: certain DNA sequences (immunostimulatory sequences: ISS) induce the production of IFN as well as the activation of NK cells. The presence of these ISS in the plasmid vector has been able to boost the immune response considerably (Sato et al., 1996; Leclerc et al., 1997) and to be responsible for non-specific protection against rabies (Bahloul et al., 1997).

Factors that influence the immune response upon DNA-based immunization

Expression upon *in vivo* transfection has mostly been studied with muscle cells, but has been obtained as well after injection of DNA into the myocardium (Lin et al., 1990) or into arterial walls (Nabel et al., 1990). The level of the immune response varies with the composition of the DNA solution and the site of injection. DNA can be administered in saline, distilled water or complexed with cationic liposomes (Hofland et al., 1996). A hypertonic solution of DNA decreases the very high variability (Wolff et al., 1991) of the immune response (Davis et al., 1993b), but the damage caused by the hypertonic solution leads to a lower level of expression. Successful transfer of plasmid DNA has been reported upon intramuscular inoculation (Wolff et al., 1990), intradermal (Tang et al., 1992; Raz et al., 1994) and intravenous injection and upon application to mucosal membranes (Fynan et al., 1993; Gao et al., 1997). Skeletal muscle seems to be the most efficient tissue for DNA take-up, because of its multinucleated cells, the transverse tubuli system and the sarcoplasmatic reticulum (Davis et al., 1993b; Wolff et al., 1990). A relaxed muscle expresses the foreign gene to a higher amount than a contracted one (Wolff et al., 1991).

Skin and mucosa assure as well an efficacious uptake of the DNA. Intradermal injection of DNA on coated gold beads using a ballistic system (Williams et al., 1991; Tang et al., 1992) has been shown to induce a very high immune response using only very little DNA (nanogram quantities per injected animal compared to 10-100 µg DNA injected intramuscularly). The skin has several advantages for DNA immunization: it is easily accessible, very rich in APCs and its high turnover minimizes the risks of integration. It has even been suggested that the immune response after intramuscular injection is due to the needle's passage by the skin (Hassett and Whitton, 1996). In contrast, no immune response has been obtained after DNA immunization into the peritoneum (Fynan et al., 1995).

The immune response after DNA immunization can be enhanced by coinjection of plasmids coding for cytokines, namely GM-CSF (Xiang and Ertl, 1995) and IL-2, that has been reported to overcome the the MHC-linked non-responsiveness to some antigens (Chow et al., 1997). A weaker immune response has been reported after

coinjection with vectors expressing IFNγ. Indeed, IFNγ seems to have an inhibitory effect on certain promoters (Xiang et al., 1997). Previous muscle degeneration that causes regeneration and increase of protein synthesis (including MHC I proteins; Karpati et al., 1988) improves the immune response (Hassett and Whitton, 1996). Besides, the subsequent local inflammation due to the degeneration also induces the migration of inflammatory cells, and with them APC to the site of injection.

Mechanism of DNA-based immunization

After uptake by the muscle cells, the DNA is translated and the foreign protein is expressed in the cytoplasm - conditions that make basically expect a processing of the protein by the MHC I pathway (Rötzschke, et al., 1991) with induction of CTL (McDonnell and Askari, 1996; Hassett and Whitton, 1996). But in many cases, the CTL response induced after DNA immunization is rather low, what is not very

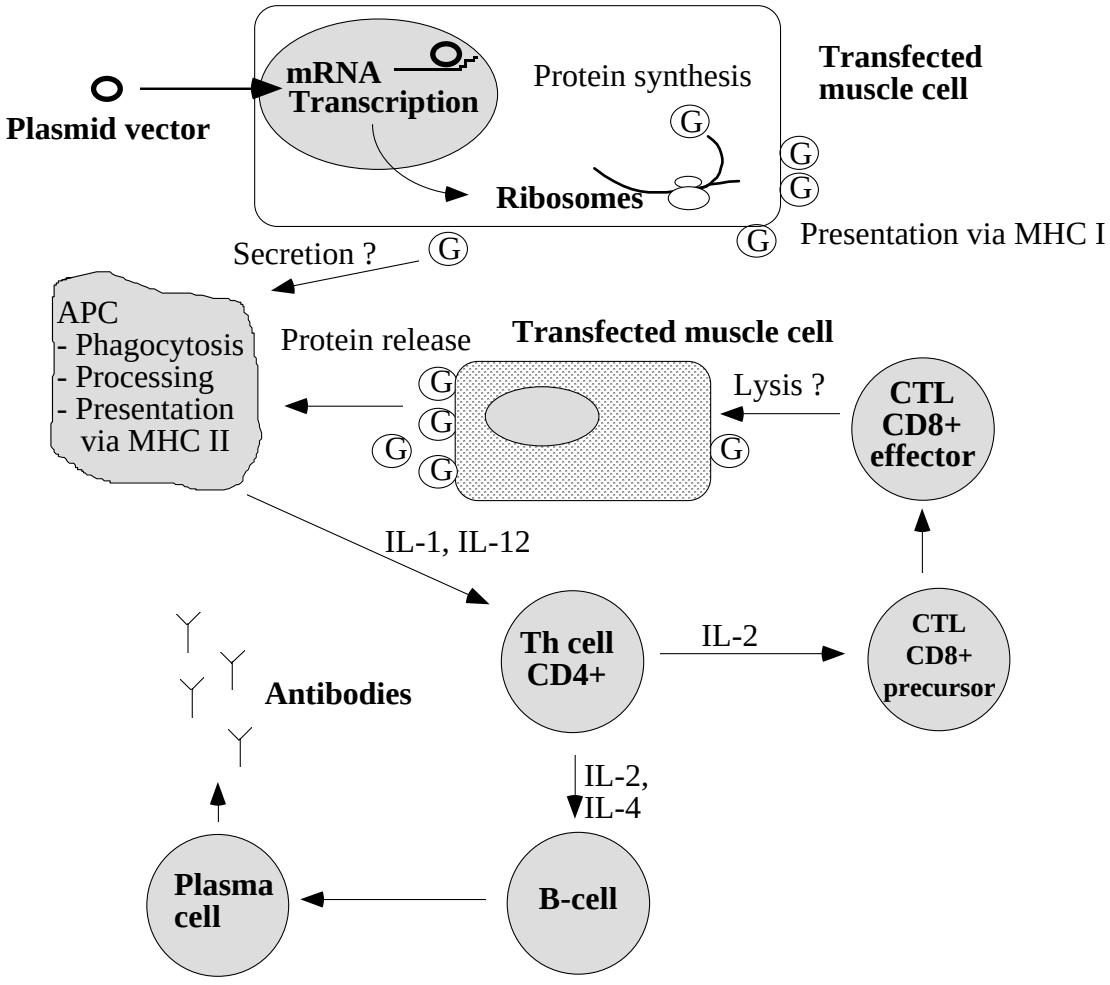


Figure I. 12.: Hypothetical mechanism of DNA-immunization.

surprising, considering that muscle cells do not carry many MHC I molecules (Karpati et al., 1988). In any case, such a presentation by muscle cells would not be involved in antibody induction since stimulation of both, B and Th cells, needs presentation by MHC II proteins that are only under exceptional conditions present on muscle cells (Hohlfeld and Engel, 1994). It could be assumed, that the muscle cells are the producers of the foreign antigen and its reservoir for spontaneous (Davis et al., 1993a) or lytic release by CTL (Hassett and Whitton, 1996; Ertl and Xiang, 1996b) and that professional antigen presenting cells are required for antigen uptake and presentation by the MHC II pathway thus allowing the antibody production (figure I. 12., p. 35).

Advantages and risks of DNA-based immunization

The advantages of DNA immunization are well known while its risks are still discussed (WHO, 1996). Production and manipulation of DNA is easy, DNA is thermostable and its production is cheaper and safer than the classical method of antigen preparation. Moreover, without injecting an infectious agent, the induced immune response comprises both B and T cell production.

Unfortunately, there are various potential risks associated with DNA-based immunization including the integration of the plasmid DNA into the host genome by homologous recombination (between the plasmid sequence and chromosomic DNA sequences of the host), by retroviruses or by random integration. The first two possibilities can be avoided since the absence of homologous sequences and retroviruses can be assured in the plasmid nucleotide sequence. Concerning random integration, it has to be stated that this phenomenon is considered to be a very rare event. So far, no indication of integration has been reported, the plasmid DNA always staying episomal, even after long term persistence (Wolff et al., 1992). Another potential risk would be induction of a tumour, by insertion of an active oncogen, by activation of a proto-oncogen or by disactivation of suppressor genes.

The long lasting immune response might be a disadvantage, as it could induce either tolerance or immunopathological events. Moreover, in rare cases, myositis caused by a CTL response after genetic immunization has been observed (Yokoyama M., 1997). One cannot exclude either that the DNA itself might induce antibodies and therefore cause auto-immune disease.

Whatever the future of the use of naked DNA as vaccine the DNA-based immunization can be considered to be a very useful technology for fundamental studies on both antigens and immune responses.

I. 2. 5. b. DNA-based immunization against rabies

DNA-based immunization has been tested for both, rabies G (Xiang et al., 1994; Bahloul et al., 1997) and N proteins (Ertl and Xiang, 1996b).

Plasmids encoding rabies G induced specific CTL and Th response as well as VNA production and protection against intramuscular (Xiang et al., 1994) and intracerebral challenges (Bahloul et al., 1997). The immune response induced is mainly a Th1 like response (IFNγ and IL-2 synthesis) that results primarily in the production of IgG2a isotypes beside IgG1 and IgG2b that are produced in lower quantities (Ertl and Xiang, 1996b; Bahloul et al., 1997). The immune response was increasing and stayed at a plateau level for the lifespan of the injected mice (Bahloul et al., 1997). It remains to be demonstrated whether longer living animals maintain the immune response in the same manner. Moreover the immune response obtained in neonatal mice was similar, indicating that DNA-based immunization would be suitable for vaccination of newborn animals (Wang et al., 1997).

In addition, the injection of a plasmid with the gene of a hybrid glycoprotein coding for the aminoterminal half of the Mokola strain and the carboxyterminal half of the PV strain induced virus neutralizing antibodies able to neutralize viruses from almost all lyssavirus genotypes showing that a "lyssavirus vaccine" can be proposed (Bahloul et al., 1997).

Concerning the N protein in DNA-based immunization, the injection of a plasmid coding for N failed to induce an immune response: the protein is not exported to the cell membrane and might therefore not be recognized by the immune system of the host (Ertl and Xiang, 1996b).

I. 3. Rabies vaccinology today and its prospects

The immunity against rabies is mainly directed against the only surface protein, the glycoprotein. An inner protein, the nucleoprotein, has been discussed to be able to prime for a following infection or vaccination. In contrast to the glycoprotein, the nucleoprotein does not vary much between the different genotypes of the lyssavirus family and it might therefore be an important element in subunit vaccines

Classical rabies vaccines consist of inactivated rabies virus but "new vaccines" against rabies have been developed as well: recombinant viruses and recombinant proteins. A recombinant vaccinia virus, expressing the glycoprotein, is used for wildlife immunization in some European countries. Recombinant proteins, produced by the baculovirus expression system can be produced in a high amount and are less expensive than whole virus vaccines, but so far, they have only experimental importance. Recently, naked DNA has been tested for immunization against rabies, presenting a very powerful and easily manipulated form of subunit vaccine.

Today's vaccines protect very well against classical rabies (genotype 1). They are used for humans, domestic animals and terrestrial wildlife (foxes). Bats, which are a reservoir of rabies virus on most continents are not reached by wildlife immunization. In Europe, terrestrial rabies might be wiped out within the next years, rising the question about the importance of the virus reservoir in the bats, especially as European bats host a lyssavirus genotype, EBL1, that is only partially covered by protection achieved by classical vaccines.

Consequently, two important questions can be risen concerning rabies vaccinology: 1) "Is there an advantage to include the nucleoprotein in a rabies subunit vaccine?"; 2) "Is it possible to develop a rabies vaccine able to protect against both rabies and European bat lyssavirus?".

II. AIM OF THE STUDY

One of the drawbacks of classical rabies vaccines is the fact that they only induce insufficient protection against classical rabies. The aim of this study was to undertake an approach towards an immunization which would induce protection against all European lyssaviruses including rabies and European bat lyssaviruses (EBL1 and 2: rabies-related viruses) which are only partially achieved by classical vaccines and which have been responsible for human deaths. To obtain a potent vaccine against all European lyssaviruses, two different approaches based on the virus glycoprotein (G) as immunogen because of its crucial role in anti-rabies immunity were investigated.

For the first approach we used the baculovirus expression system for the production of rabies antigens including the G protein and the internal nucleoprotein (N). As N has been reported to induce cross-protection and to have adjuvant properties in rabies subunit vaccines, this protein is a natural candidate for a vaccine with broadened spectrum even though the precise role of N in rabies immunity is still object of controversy. "Virologically pure" rabies G and N proteins were produced by the baculovirus expression system and N protein was tested for both, its adjuvant and/or priming properties (including a a cross-help effect) for a G+N antigen combination and for its capability to improve protection against EBL viruses.

The second approach employed DNA-based immunization which consists in direct injection of a eukaryotic expression plasmidic vector containing the gene of the immunogen. This method has recently been tested using a chimera of the Mokola and the Pasteur virus (PV) glycoproteins (Mok/PV) which induced protection against a large spectrum of lyssaviruses but poorly covered EBL1 virus (Bahloul et al., 1997). In order to induce protection against this virus, we have constructed a similar chimera (EBL1/PV) containing the aminoterminal half of the EBL1 glycoprotein with the antigenic site II and the carboxyterminal half of the PV glycoprotein with the antigenic site III. Immunological responses and protection against both EBL1 and PV viruses were studied. Furthermore, a plasmid coding for a truncated rabies glycoprotein, containing only the carboxyterminal half with the antigenic site III has been constructed and it has been investigated which immunological functions of the "full" glycoprotein are maintained by this polypeptide. The possibility to insert foreign epitopes in pEBL1/PV or pPVIII in order to obtain a transdisease vaccine will be discussed.

Moreover, with the goal to investigate whether a relation between *in vitro* expression and immunological function *in vivo* of the rabies glycoprotein can be determined, we have analysed the expression of plasmids coding for different glycoprotein constructions and compared these results to the immune response obtained after DNA-based immunization.

MATERIALS AND METHODS

III. MATERIALS AND METHODS

III. 1. Animals

Animal experiments have been carried out on mice (Janvier). For antibody and IL-2 production female, 6-7 weeks old Balb/C mice (haplotype H-2^{dd}) or 6-7 weeks old C3H/He mice (haplotype H-2^{kk}) have been used. Protection was tested on 6-7 weeks old female Balb/c mice or on male OF1 (no inbred line) mice with a body weight of 14-16g. Each immunization group consisted of 2 to 5 animals.

III. 2. Cell culture

Cell culture media

(For details concerning culture mediums and buffers see appendix III. 1.).

• *RPMI medium:*

RPMI medium is used for CTLL cell (see below) and mouse splenocyte culture.

•*RPMI medium for washing:*

For cell washing RPMI is supplemented with glutamine and antibiotics.

•*Complete RPMI medium:*

For cell culture RPMI medium for washing is supplemented with fetal calf serum (FCS), aminoacids, sodium pyruvate and β-mercaptoethanol.

•*Eagle's minimum essentiel medium (MEM):*

For culture of BHK-21 and Neuro-2a cells MEM is supplemented with tryptose phosphate buffer, glutamine and in some cases with antibiotics.

•*TC100:*

TC100 has been kindly supplied by Otto Merten (Pasteur Institute). It is used for culture of Sf9 cells (see § III. 2. 3.) after supplementation with 10% FCS.

• *Express Five SFM (Gibco BRL)*:

Express Five SFM (serum free medium) is used for culture of High5 cells.

Cells

•*BHK-21 C13 (ATCC: CCL 10):*

BHK-21 cells clone 13 (Baby Hamster Kidney) (MacPherson, et al., 1962) are used for rabies virus production and virus titration as well as for titration of neutralizing antibodies. They are cultivated at 37° C in monolayer culture in MEM-medium which has been supplemented with tryptose phosphate, glutamine and 5% FCS (Flow or Gibco) and 5% of new-born calf serum (Flow or Gibco) (Perrin, et al., 1982). Twice a week they are passaged after dissociation with trypsine-versene solution.

•*Neuro-2a (ATCC: CCL 131):*

Neuro-2a are grown in monolayer at 37° C with MEM-TPB-Glu 10% FCS (Flow or Gibco). Twice a week they are passaged after dissociation with trypsine-versene solution.

•*Cytotoxic T lymphoid line (CTLL-2: AATCC-TIB 214):*

The CTLL are derived from cytotoxic T cells from mice. They are used for IL-2 quantification as their survival and growth depends on IL-2. The CTLL-2 cells (0.5 - $2x10^4$ cells / 5 ml) are grown in suspension at 37° C with CO_2 in complete RPMI medium containing 5-10 units per ml of rat IL-2. They are passaged twice a week.

•*Trichoplusia ni cells (High5: BTI-TN-5B1-4, GibcoBRL) and Spodoptora frugiperda cells (Sf9, Pharmingen):*

Both cell lines are grown in monolayer at 28° C in Express Five SFM or TC100 10% FCS medium respectively. They are passaged twice a week after mechanic dissociation by tapping on the culture vessel or by pipetting vigorously.

III. 3. Virus strains and viral antigens

Virus strains

PV-Paris/BHK

The Pasteur virus (PV) strain has been isolated by Pasteur in 1882. After numerous passages on rabbit brain the strain has been adapted and amplified on BHK-21 C13 cells and purified to obtain high virus titres (Perrin et al., 1982). The strain PV-Paris/BHK/purif.pass.4 with a titre of $1.2x10^9$ fluorescent focus units per ml (FFU/ml; see § III. 4.) is used for rabies antigen production. Clarified viruses from infected cell supernatants, purified viruses or inactivated and purified viruses (IPRV) were prepared as previously reported (Perrin, 1996a).

CVS:

The Challenge Virus Strain (CVS) has been obtained in the United States by passage of the strain PV first on mouse brains and subsequently on BHK cells. The strain is used as challenge virus for virus neutralizing antibody (VNA) titration and for control of protection achieved by rabies vaccines.

European bat lyssavirus (EBL1):

The EBL1 strain used as challenge virus for VNA titration and for control of protection has been obtained by amplification on BHK cells. Clarified viruses from

infected cell supernatants, purified viruses or inactivated and purified viruses (IPRV) were prepared as previously reported (Perrin, 1996a).

Mokola (Mok):

The Mokola strain used as challenge virus for VNA titration has been prepared in the same manner as EBL1 virus (§ III. 3. 2.).

Viral antigens

Inactivated purified rabies virus (IPRV):

The supernatant of infected BHK-21 cells is clarified, inactivated with 1/4000 β-propiolactone (Sigma), concentrated by ultracentrifugation (Beckman J14, 19000 g, 8 h) and purified by centrifugation (Beckman SD28, 80000 g, 120 min) on a sucrose gradient (20-50%).

Ribonucleoprotein (RNP):

RNP is extracted from infected BHK cells and purified following the method described by Compans et coll. (Compans and Choppin, 1967) modified by Sokol (Sokol, 1973a). Briefly, the cells are lysed by osmotic shock, the lysate is clarified by centrifugation (Beckman J17, 5000 g, 10 min) and the supernatant is purified on CsCl gradient (2 g CsCl for 4.5 ml supernatant; Beckman SW50, 150000 g, 22h).

Glycoprotein (G):

G is extracted from purified virus following the method described by Perrin et coll. (Perrin et al., 1982; Perrin et al., 1985b). Briefly, G is extracted with 2% octylglycopyranoside (OGP, Sigma) in Na-Tris-buffer (NT) at normal temperature for 45 min and subsequently centrifuged on a 25% sucrose cushion (Beckman SW50, 150000 g, 100 min) and purified on a sucrose gradient (5-25%) containing 2% OGP. Finally the protein solution is dialysed against phosphate buffer saline (PBS).

III. 4. Virus titration

Rabies virus

Rabies virus is titrated using a modified RFFIT technique (see § III. 11.). Briefly, the virus suspension is diluted in microplates, BHK cells ($4x10^4$ per well) are added and after 24 h incubation the virus dilution that induced 1 fluorescent focus is determined and the virus titre is expressed as the inverse of that dilution (fluorescent focus units (FFU)/ml).

Recombinant baculovirus

Recombinant baculovirus is titrated as well following a modified RFFIT technique. The virus suspension is diluted in 96 well plates. $5x10^4$ Sf9 or High5 cells are added per well. The plate is covered and incubated at 28° C for 2 days. The fluorescent foci are determined by indirect immunofluorescence (see § III. 6.) with the anti-glycoprotein PV serum (rabbit) for detection of recombinant glycoprotein (G) and with the purified anti-RNP PV antibody coupled to fluoresceine isothiocyanate (FITC) for detection of recombinant nucleoprotein (N). The virus titre corresponds to the inverse of the dilution that contained 1 fluorescent focus unit FFU.

III. 5. Protein quantification and analysis

BCA

Protein concentration in the antigen preparations is measured with the Micro BCA (bicinchoninic acid) reagents (Pierce Rockford, IL USA 61115) following the method of Smith et coll. (Smith, 1985). Bovine serum albumin (Fraction V, Sigma) is used as reference.

SDS-polyacrylamide gel electrophoresis and Western blot

Proteins are separated according to their size in an electric field under denaturing conditions. The proteins are then transferred on a nitrocellulose membrane and antigens are detected with specific antibodies.

The proteins are denatured with 1 volume of Laemmli buffer (0.5 g trizma base, 0.4 g sodium dodecylsulfate (SDS), 1.0 ml 2-mercaptoethanol, 2.0 ml glycerol, 7.0 ml H_2O, 0.02 g bromophenol blue) during 4 minutes at 100° C. Electrophoresis is run on a separated gel: 1) for 20 ml resolving gel: 9.3 ml H_2O, 5.3 ml 30% acrylamide mix (29/1 acrylamide/bisacrylamide - Bio-Rad), 5 ml 1.5 M Tris pH 8.8, 0.2 ml 10% SDS, 0.2 ml 10% ammonium persulfate, 12 µl Temed - Bio-Rad, 2) for 5 ml stacking gel: 3.4 ml H_2O, 0.83 ml 30% acrylamide mix, 0.63 ml 1 M Tris pH 6.8, 0.05 ml 10% SDS, 0.05 ml 10% ammonium persulfate, 5 µl Temed. The electrophoresis buffer is composed as follows: 3.027 g Tris, 14.4 g glycine, 5 ml 20% SDS, H_2O ad1 l.

For protein staining the gel is immersed in Coomassie Blue solution (450 ml methanol, 90 ml acetic acid, 2.5 g Coomassie Blue, 450 ml H_2O) for 1 hour and destained overnight in destaining solution (520 ml H_2O, 400 ml methanol, 80 ml acetic acid). For immunoblotting the gel is transferred in transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol) on nitrocellulose (Schleicher & Schuell) using a semi-dry electrotransfer unit (Sartoblot II). For immunostaining the membrane is saturated for three hours at 4° C in PBS containing 0.05% Tween 20 and 5% BSA.

The diluted antibody (rabbit anti-G PV or anti-RNP PV) is added and the incubation continued overnight. The membrane is then washed with H_2O and immersed for 10 min in PBS. This procedure is repeated twice. The membrane is again washed with water and incubated with the antiserum (goat anti-rabbit alkaline phosphatase conjugate, Bio-Rad) at 4° C for 1 hour and washed again in the same manner. The membrane is equilibrated for 5 min in alcaline buffer (100 mM NaCl, 50mM $MgCl_2$, 100 mM Tris HCl pH 9.5, 0.1% Tween 20) and stained with staining buffer (10 ml of alcaline buffer, 45 µl nitroblue tetrazolium chloride, 35µl 5-Bromo, 4-Chloro, 3-Indolylphosphate (Sigma)). The reaction is stopped by washing with H_2O.

III. 6. Antigen quantification and analysis of expression

ELISA

The ELISA method is based on the antigen-antibody-reaction. An antigen is immunocaptured by an antibody and stained by the same antibody coupled to peroxidase. This technique is used for the quantification of G, of recombinant N and of viral RNP.

Polyclonal antibodies are produced in rabbits, purified and coupled to peroxidase as described (Perrin et al., 1985a). Anti-glycoprotein antibodies are obtained after immunization of rabbits with glycoprotein preparations of Pasteur Virus produced on BHK cells. Anti-ribonucleoprotein antibodies are obtained after immunisation of rabbits with purified RNP produced on PV infected BHK-21 cells.

The ELISA test (Perrin et al., 1996a) is carried out in a 96 well microplate (Nunc Maxisorp). The immunosorbent (anti-G or anti-N antibodies) is diluted in carbonate-bicarbonate buffer pH 9.6 (for composition of buffers see appendix), distributed in a volume of 200 µl per well and incubated in a humid atmosphere for 3 h at 37° C and for 18 h at 4° C. The wells are emptied, refilled with 300 µl of stabilization solution and incubated at 37° C for 30 min. The microplates are washed with PBS-Tween buffer pH 4.7. The antigens are diluted on ice in PBS-Tween-BSA buffer. 200 µl are distributed in the wells and incubated at 37° C for 1 hour. The microplate is washed again and 200 µl of antibody coupled to peroxidase are distributed per well. The microplate is incubated at 37° C for 1 hour and washed. The peroxidase activity is demonstrated by adding 200 µl of chromogen substrate (o-phenylenediamine, OPD). The plates are incubated at room temperature in the dark for 30 min. The staining is stopped by addition of 50 µl per well of H_2SO_4 4 N and the optical density at 492 nm is measured. The results are expressed in relation to a reference curve obtained with a known antigen solution.

As reference we use either an IPRV preparation for G quantification or purified RNP for N quantification.

Indirect immunofluorescence

A cellular antigen is demonstrated by fixation of a specific antibody and an anti-antibody (anti-species) coupled to fluorescine isothiocyanate (FITC) and binding specifically to the first antibody.

Cells are grown in a 96 well plate. They are washed twice with PBS Ca^{2+}/Mg^{2+}. For fixation, 100 µl of 80% chilled acetone are added per well. The plate is incubated on ice for 30 min. The wells are emptied, and the acetone is permitted to evaporate. For non-fixed cells, staining is started immediately after washing the cells.

The first antibody is diluted in PBS. 50 µl of the solution are added per well, the plate is covered and incubated at 37° C for one hour. The cells are washed twice with PBS. The second antibody (anti-species) coupled to FITC is diluted in PBS together with Evans Blue (0.05%). 50 µl of the solution are added per well, the plate is covered and incubated at 37° C for one hour. The cells are washed twice with PBS and 50 µl of 50% glycerol/PBS are added per well. Fluorescence is evaluated by microscope.

Purified antibodies and sera used for indirect immunofluorescence on insect cells and Neuro-2a:

- polyclonal (poly) anti-G PV antibody, rabbit
- polyclonal anti-G Mok antibody, rabbit
- polyclonal purified antibody anti-IPRV EBL1, mouse
- purified monoclonal (mono) antibody anti-site III of PV glycoprotein, mouse (D1)
- monoclonal purified antibody anti-site II of PV glycoprotein, mouse (PVE12, Monique Lafon, Institut Pasteur)
- monoclonal purified IgG specific for aminoacid (aa) 426 - aa 452 of PV glycoprotein, mouse (6-B1, Yves Gaudin, CNRS, Gif sur Iyette): the 6-B1 site is only accessible on rabies G during early stages of synthesis or on denatured G.

Anti-species antibodies:

- anti-IgG mouse FITC (Nordic)
- anti-IgG rabbit FITC (Nordic)

III. 7. Preparation of recombinant rabies proteins

Production of recombinant baculoviruses*

The principle of the baculovirus expression system is described in the introduction (§ I. 2. 3. b). In order to transfer glycoprotein and nucleoprotein gene (from PV rabies virus) into the *Autographa californica Nuclear Polyhedrosis Virus* (AcNPV) genome, RT-PCR products were subcloned and inserted under the control of the polyhedrin promoter into the Baculovirus transfer vector pVL1393 (Pharmingen). The distance between the start site of polyhedrin mRNA transcription and the initiation codon of both genes was reduced to a a few nucleotides by using degenerated PCR primers with artificial restriction sites. Recombinant baculoviruses were isolated after cotransfection of Sf9 cell with linear, non infectious AcNPV DNA (BaculoGold^{TM}) and recombinant transfer vector. Clonal selection of pure recombinant baculovirus was carried out by plaque-assay purification.

Production of recombinant rabies glycoprotein in insect cells

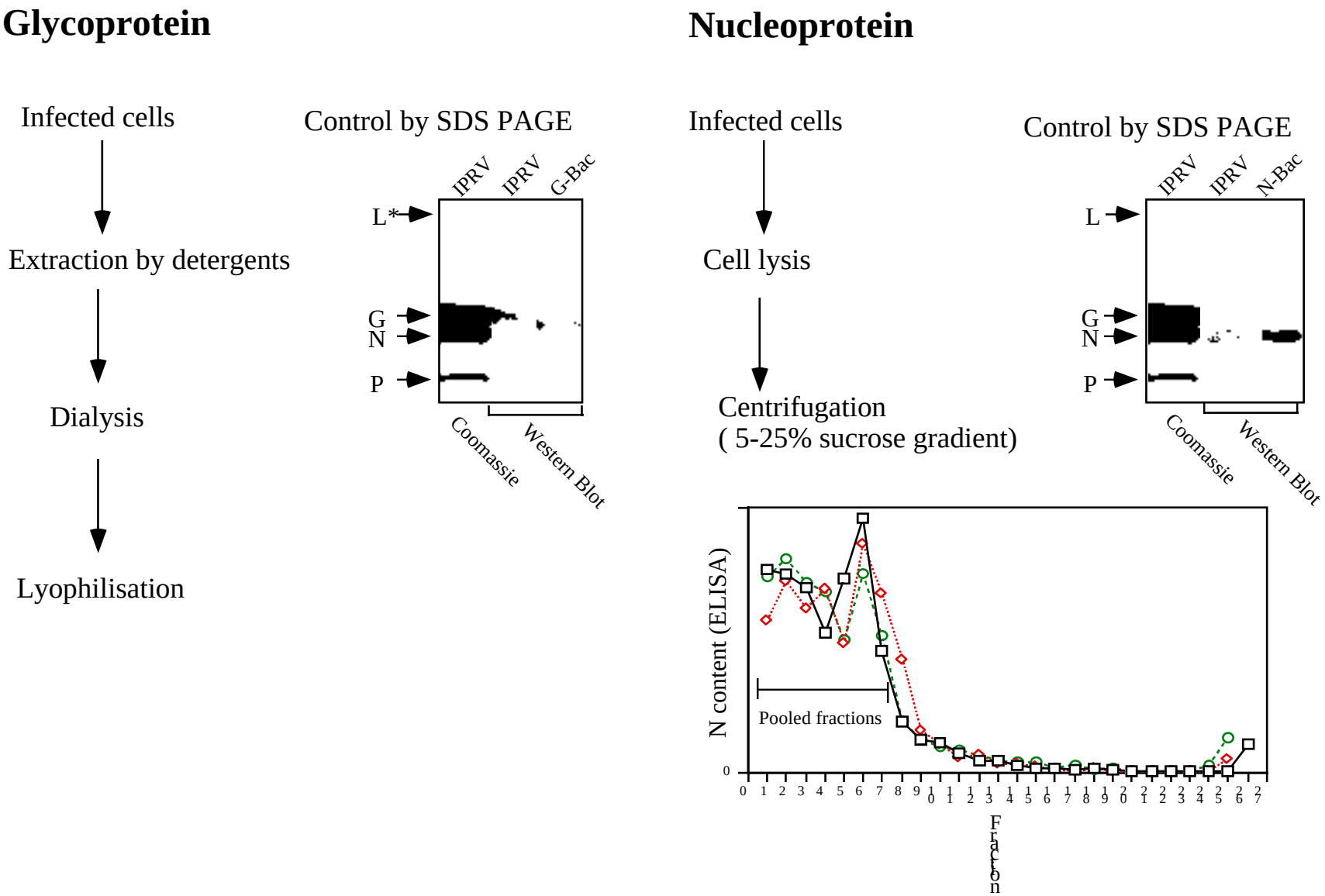
General procedure(see figure III. 1.):

Sf9 or High5 cells are infected in suspension at a MOI (multiplicity of infection) of 1 FFU G recombinant baculovirus per cell. For the harvest of the infected cells, they are dissociated by tapping on the cell culture flask, the cell suspension is centrifuged for 5 min at 1500 rotations per minute (rpm), the supernatant is decanted and stored at 4° C. If it is to be used as antigen (G supernatant, Gs-Bac) protease inhibitor is added (Complete, Boehringer Mannheim). The cells are washed with PBS Ca^{2+}/Mg^{2+}, a protease inhibitor is added and the cells are stored at -20° C.

For extraction of recombinant G (G-Bac) a detergent is added. Different concentrations of octylglycopyranoside (OGP, Sigma), Triton X (Sigma) or Chaps (Sigma) have been tested and the best result has been obtained with 1% chaps. The cell suspension is incubated at room temperature for 1 hour and repeatedly mixed. The suspension is centrifuged for 10 min at 15000 rpm (Sigma 2K15). The supernatant is decanted, G is quantified by ELISA and the solution is dialysed in dialysis membranes (Spektrapur): for preparation of dialysis membranes they are boiled in H_2O, rinsed in H_2O and stored in 0.15 M disodium EDTA solution; before use the membranes are plunged in a 20% glycerol 0.01 M Tris pH 7 solution for 1 hour at 4° C and rinsed with H_2O. The solution that is to be dialysed is filled in the membranes and dialysed for 12-24 hours against NT buffer containing 1 mM NaN_3 and 1/1000 polymethylsulfonilfluoride (PMSF, Sigma). G-Bac is

* Recombinant baculoviruses have been prepared by the molecular biology group of the lyssavirus laboratory (Y. Jacob and coll.)

Figure III. 1.: Preparation of recombinant rabies proteins.
* L = Polymerase; G = Glycoprotein; N = Nucleoprotein; P = Phosphoprotein (the matrix protein is not visible on this electrophoresis).



quantified by ELISA. As in spite of the addition of protease inhibitors the recombinant G has been shown not to be stable at 4° C, the protein solutions are lyophilized. Furthermore, concentration is necessary for encapsulation in liposomes (see below). Recombinant G is controlled by Western Blot (see § III. 5.).

In order to assure that the amount of glycoprotein measured by ELISA corresponds to an immunological functional protein, G-Bac extracted with 1% chaps has been tested *in vivo* . Balb/c mice were injected intraperitoneally at day 0 and day 7 with 0.3 µg of G-Bac. The mice were bled at day 20 and VNA against CVS were titrated. The mice had developed VNA at titres of 2,2 and 4,5 IU/ml. It can be concluded that G-Bac extracted with chaps is immunologically potent.

Choice of cell lines and harvest time for virus and recombinant glycoprotein production:

Sf9 cells and High5 cells have been compared for their suitability to produce recombinant baculovirus and recombinant G protein. Both cell lines produced the same amount of Gs-Bac and of G-Bac extract. Recombinant virus production was up to 1000 x higher by Sf9 cells than by High5 cells with a maximum at 48 h. Sf9 cells were therefore chosen for recombinant virus production whereas recombinant proteins were produced by High5 cells as they grow in serum-free medium and thus reduce the contamination of recombinant G by foreign proteins.

The optimal harvest time for G-Bac production by High5 cells has been determined at 96 h. A maximum of Gs-Bac production has been observed at 72 h.

Production of recombinant rabies nucleoprotein in insect cells

(see figure III. 1.)

Cells are infected in the same manner as for G-Bac production with N recombinant baculovirus. They are harvested after 48 h. After centrifugation, they are lysed with H_2O, centrifuged and the supernatant is dosed on ELISA for its content of N-Bac. N-Bac is purified on a sucrose gradient: a centrifugation tube (Polyallomer, Beckman) is filled with 0.5 ml of 65% sucrose/NT solution and overlayered by a gradient of 4.5 ml 60% sucrose/NT and 5.25 ml 5% sucrose/NT. 1 ml of Sf9 lysate containing N-Bac is added per gradient. The tube is centrifuged at 35000 rpm for 39 h at 4° C (L7-65 Ultracentrifuge, Beckman, SW41). Fractions of 500 µl are collected and N-Bac is dosed by ELISA (see § III. 6.). The fractions containing N-Bac are pooled (figure III. 1.) and stored at -20° C. Recombinant N is controlled by Western blot.

Protein encapsulation in liposomes

For encapsulation of the proteins in liposomes a phospholipidic film (Bangham, 1965) is obtained by mixing 6.25 mg of egg lecitine (Avanti) and 1.5 mg of

cholesterol (Avanti) dissolved in chloroform/methanol (V/V) in a rotating evaporator connected to a refrigerating system and a vacuum pump. The protein solution is added to the phospholipidic film in a ratio of 65 µg protein per mg lipids. The obtained proteoliposomes are stored at 4° C. All operations are carried out in glass recipients that have been thoroughly washed with 50/50 chloroform/methanol.

For proteoliposomes that contain both N and G, the protein solutions are mixed before adding to the phospholipidic film.

III. 8. Plasmid preparations and control

GENERAL TECHNIQUES

For plasmid preparation we have used the classical methods of molecular biology (Maniatis et al., 1982). If not mentioned differently, the chemical agents were supplied by Prolabo, GibcoBRL or Sigma.

Preparation of competent bacteria preparation of for electroporation

E. coli DH5αF are grown overnight in preculture in 100 ml Luria Bertani medium (LB; for bacteria culture meda see appendix). 2 litres of LB are inoculated with the preculture in order to obtain an optical density $(OD)_{600}$ of 0,01-0,05 and bacteria are grown to a maximal OD_{600} of 0.5. The culture medium is rapidly chilled on ice and centrifuged during 5 minutes at about 4500 rpm. The pellet is washed three times with chilled distilled water and once with chilled 10% glycerol. Finally the pellet is resuspended in 0.5 ml of 10% glycerol. The bacteria are dispatched in volumes of 70 µl and stored at -80°C.

Bacteria transformation by electroporation

Competent bacteria are thawed and DNA solution (ca. 1 µg in less than 5 µl) is added. The solution is transferred into a chilled electroporation cuvette (EuroGentech) and exposed to an electric shock of 2,5 kV 25 mF 200 Ohms (Easyject, EuroGentech). 1 ml of liquid LB is added. After 30-45 min of incubation at 37°C the solution is spread on solid LB with ampicilline.

Plasmid amplification

Transformed DH5 are amplified in LB or Terrific Broth. The plasmid is purified using the QIAGEN purification kit and quantified by measuring the OD_{260}, given that an OD_{260} of 1 corresponds to a DNA solution of 50 µg/ml. The plasmid is stored at minus 20°C in H_2O.

DNA precipitation

For DNA precipitation 1/10 volume of sodiumacetate 3 M pH 5.2 and 3 volumes of absolute ethanol are added to the DNA solution. The solution is centrifuged (Sigma 2K15) at 15000 rpm for 30 min, decanted and vacuum-dried.

If the DNA is prepared for ligation, the precipitation is carried out by adding 1 volume of ammoniumacetate 5 M, and 3 volumes of absolute ethanol. The solution is then centrifuged at 15000 rpm for 30 min, decanted and vacuum-dried.

DNA gel elecrophoresis

The DNA sample is mixed with bromophenol blue (6x solution: 0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol) and run in 1-1.5% agarose (GibcoBRL) gel in 1X Tris-borate EDTA buffer (TBE: 12.114 g Tris, 5.66 g boric acid, 0.585 g EDTA, H_2O ad 1 l; spontaneous pH) in an electric field of 6 V/cm for 30-45 min. After migration the gel is coloured in an ethidium bromide (Sigma) bath (0.5 µg/ml TBE).

DNA digestion

The Biolabs buffers for restriction enzymes (NBE I-IV) are used following the recommendations of the manufacturer. Approximately 1 unit (U) per µg substrate DNA are incubated for 1 hour in a final volume of 20-50 µl at the temperature indicated by the fabricant. The restriction enzymes used are: Bcl I (Biolabs), EcoR I (Boehringer Mannheim), BstE II (Biolabs), Sma I (Biolabs).

DNA ligation

6 u T4 DNA-Ligase (Pharmacia) are used in a final volume of 10-20 µl with "one for all" ligation buffer (Pharmacia) and 0.1 mM ATP and an insert/vector relation of about 5/1. The reaction is incubated overnight at 16°C.

PCR

• *PCR mix:*

5 µl 25mM $MgCl_2$, 10 µl 10X reaction buffer PCR (100 mM Tris-HCl pH 8,3 at room temperature, 500 mM KCl, 0,1 % gelatine), 16 µl dNTP (Pharmacia) 1,25 mM each NTP, 5 µl primer upstream and 5 µl primer downstream each at 15 mM in 10 mM Tris-HCl pH 8.3, 1 µl Taq Polymerase (Roche Cetus, 5U/µl), H_2O ad 100 µl. To each PCR matrix 100 µl of PCR mix are added. For direct colony screening each colony is resuspended in 25 µl of PCR mix.

• *Amplification reaction:*

PCR is done on Biometra Unothermoblock: after a hotstart of 30 seconds at 94°C 30 cycles of 30 s at 94° C, 30 s at 50° C, 60 s at 72° C are run.

DNA purification on acrylamide-bisacrylamide gel

The DNA is loaded on a 4% acrylamide-bisacrylamide gel (5 ml 10X TBE, 500 µl ammoniumpersulfate, 6.7 ml 30% acrylamide mix (29 g acrylamide, 1 g bisacrylamide, H_2O ad 50 ml, 50 µl Temed and migrated in an electric field of 5 V/cm for 30-45 min. The DNA band is cut out, crushed, resuspended in 500 µl TE and eluted by simple diffusion at 37°C overnight. The solution is centrifuged for 2 minutes at maximal speed, the supernatant is collected immediately and the DNA is precipitated.

Screening of bacteria clones by bacterial lysis

Each tested colony is incubated with 25 µl "cracking buffer" (50 mM NaOH, 0,5% SDS, 5 mM EDTA, as much bromocresol green as to obtain a light colour) at 65°C for 35 minutes. The sample is submitted to electrophoresis in a 1% agarose gel at 6 V/cm for 45-60 min. Supercoiled plasmid DNA is analysed for insertion by comigration with empty plasmid vector.

Sequencing

For sequencing the ABI PRISM 377 Dye Terminator is used (Cyclo Sequencing Ready Reaction Kit, Perkin Elmer).

• *Sequencing reaction:*

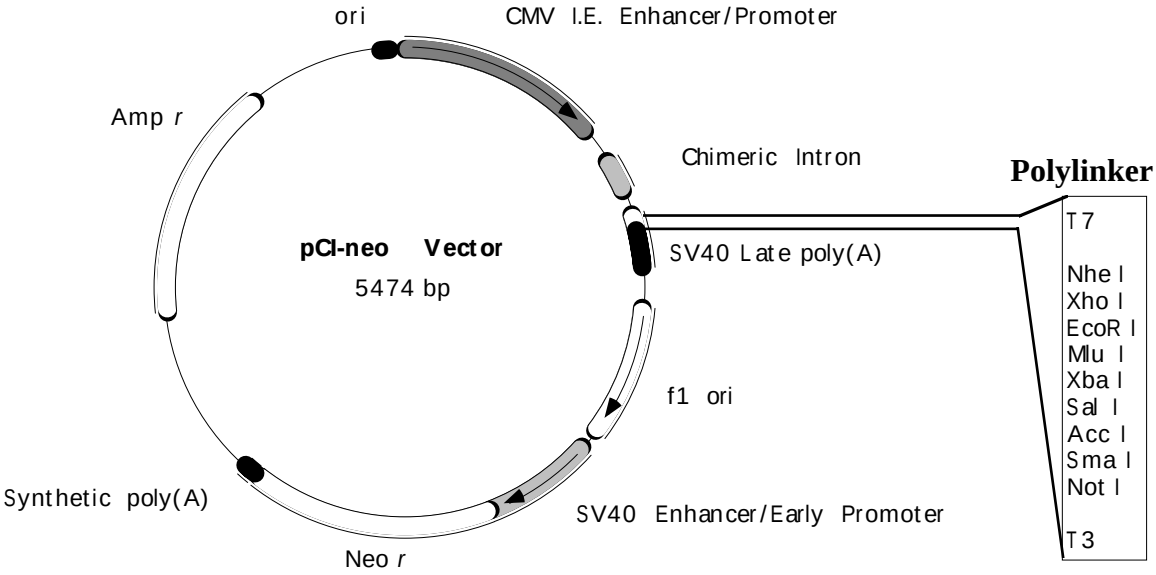
8 µl DNA (ca. 1 µg), 4 µl primer 0,8 pmol/µl, 8 µl Terminator Premix (A-Dye Terminator, C-Dye Terminator, G-Dye Terminator, T-Dye Terminator, dITP, dATP, dCTP, dTTP, Tris-HCl (pH 9.0), MgCl2, thermal stable pyrophosphatase, and AmpliTaq DNA Polymerase, FS).

• *Sequencing Cycle :*25 cycles: 10 s 96° C, 5 s 50° C, 4 min 60° C.

The amplified product is precipitated with sodiumacetate and 5 µl DNA carrier (0.25% polyacrylamide (Bio-Rad) in H_2O). The DNA is washed with 0,5 ml 70% ethanol and resuspended in 6 µl loading buffer (deionized formamide and 25 mM EDTA pH 8.0 containing 50 µg/ml Blue dextran in a ratio of 5:1 formamide to EDTA/Blue dextran).

• *Sequencing gel:*

A solution containing 28.8 g urea, 8.8 ml 40% acrylamide mix (38 g acrylamide, 2 g bisacrylamide, 100 ml ED) and 30 ml H_2O is deionized by adding 1 g resine (Bio-Rad) and agitating with a magnetic barrel for 15 min. 10X TBE buffer and 500 ml ammoniumpersulfate are filtered on Nalgene filter units. The acrylamide solution is then filtered. Polymerization of the filtered solution is induced with 70 µl Temed. After polymerization the gel is charged with 1.5 µl of the DNA loading buffer solution. Electrophoresis is run on ABI Prism 377 (Perkin Elmer).



A foreign gene expressed by the mammalian expression vector pCI-neo is under the control of the CMV I.E. enhancer/promotor.

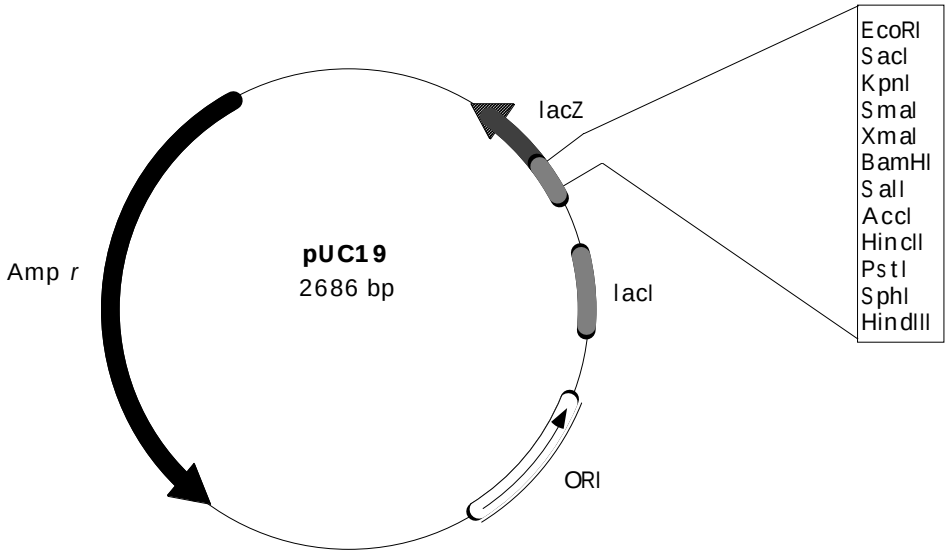


Figure III. 2.: Plasmid vectors pCI-neo and pUC.

PLASMID CLONING

Cloning of pPVIII

• *Summary (figure III. 3.):*

A pCI-neo expression vector (Promega; figure III. 2.) coding for a fusion protein G-Mok/PV (pMok/PV; appendix III. 2.), was available in the laboratory (Bahloul et al., 1997). By digestion with Bcl I and EcoR I the nucleotides coding for the aminoterminal half of G-Mok/PV are cut out, leaving a truncated carboxyterminal half containing site III of PV. The gap is filled by an oligonucleotide, "adaptor träger", coding for the peptide signal of PV and the antigenic sites b+c of PV, therefore restoring the full carboxyterminal half of GPV (PVIII). The "adaptor träger" contains a EcoR I restriction site between the signal peptide and sites b+c (introduction of one amino acid that is not present in the natural protein). The EcoR I restriction site on the vector that has been generated by the digestion is destroyed by the insertion.

• *Vector PV site III:*

pMok/PV is digested in two steps by Bcl I and EcoR I. Proteins are extracted by one volume phenol/chloroform (1 volume phenol and 1 volume chloroform: 24/1 isoamylic alcohol pH 7.5). The solution is mixed thoroughly and centrifuged for 5 min at 5000 rpm (Sigma 112). The upper phase is collected and the extraction repeated. Phenol is removed from the upper phase by 3 extractions with H_2O saturated ether: 2 volumes of ether solutions are added, the solution is mixed carefully and centrifuged for 1 min at 5000 rpm. The upper phase is removed. The DNA is precipitated with ammoniumacetate and resuspended in 25 µl H_2O.

• *Annealing and insertion of „adaptor träger:*

200 pmol of each oligonucleotide „träger-up and „träger-down (appendix III. 5.; Genset) are frozen and then vacuumdried in an Eppendorf tube during one hour. The DNA is resuspended in 10 µl of H_2O and 50 µl of PCR oil (mineral oil) are added. The tube is incubated at 95°C for 5 min, cooled down to room temperature and put on ice. After ligation with the vector the DNA is precipitated with ammoniumacetate and resuspended in 15 µl H_2O. Bacterial transformation is carried out with 5 µl of DNA solution. The bacterial colonies are screened by PCR using the primers "pCIn 5" and "träger control" (see appendix III. 6.). For sequencing the DNA is purified using the Wizzard Miniprep (Promega) and sequenced using the primers "pCIn 5" (up) and "träger control" (down). The correct clones are amplified for *in vitro* and *in vivo* analysis (Structure of PVIII: appendix III. 1., sequence of open reading frame of pPVIII: appendix III. 3., sequenced part of pPVIII: appendix III. 7).

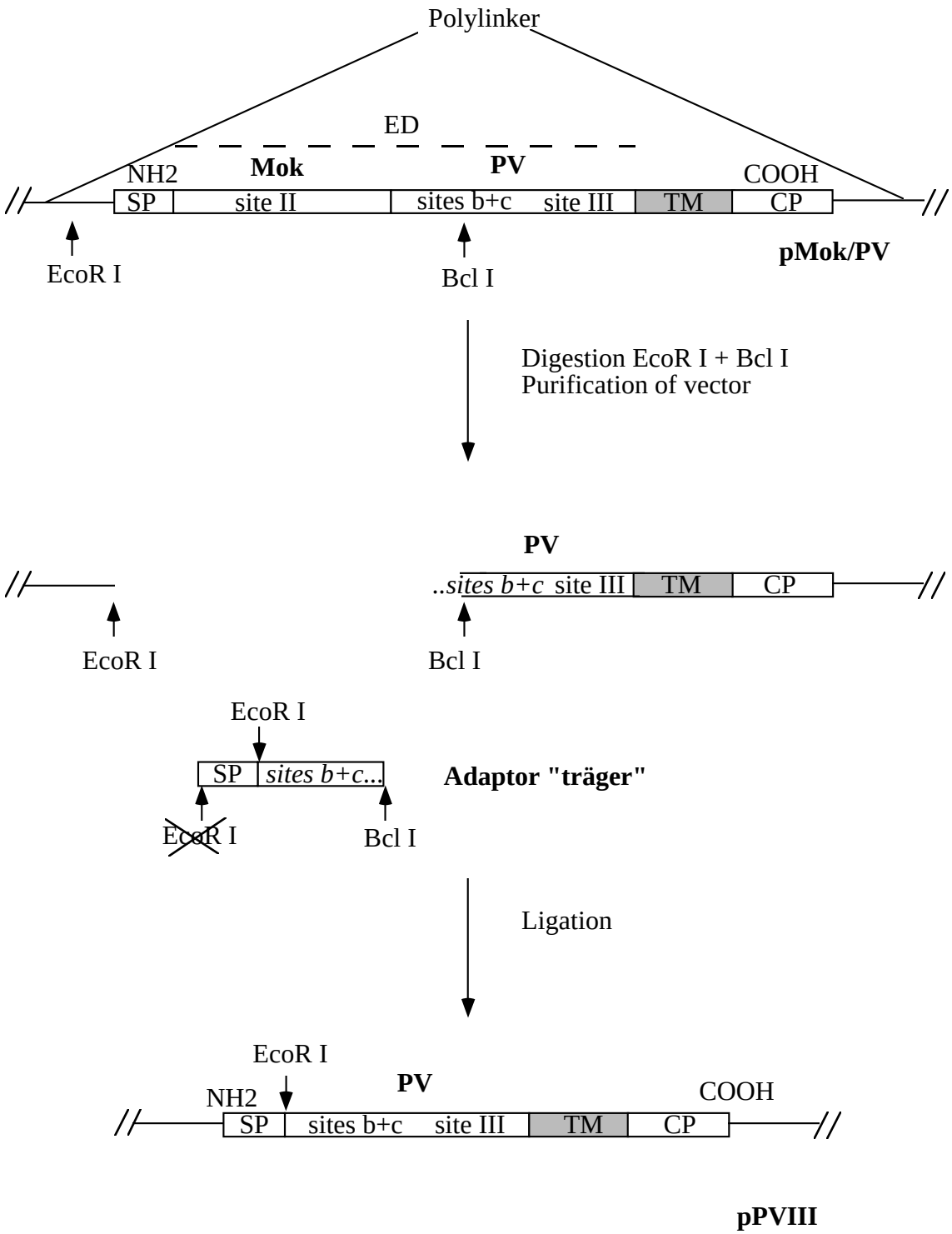


Figure III. 3. Cloning of pPVIII (SP=Signal peptide, ED=Ectodomain, TM=Transmembrane domain, CP=Cytoplasmic domain).

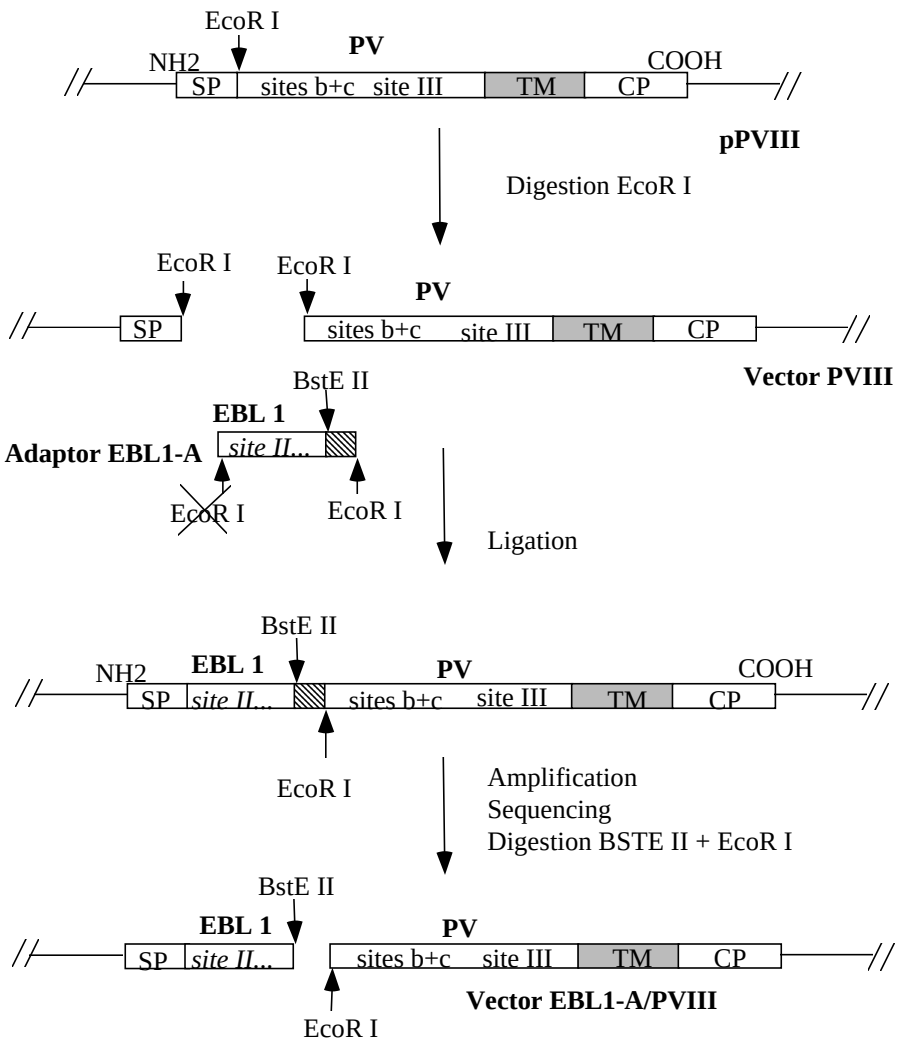


Figure III. 4. First step of cloning of pEBL1/PV: Vector EBL1-A/PV.

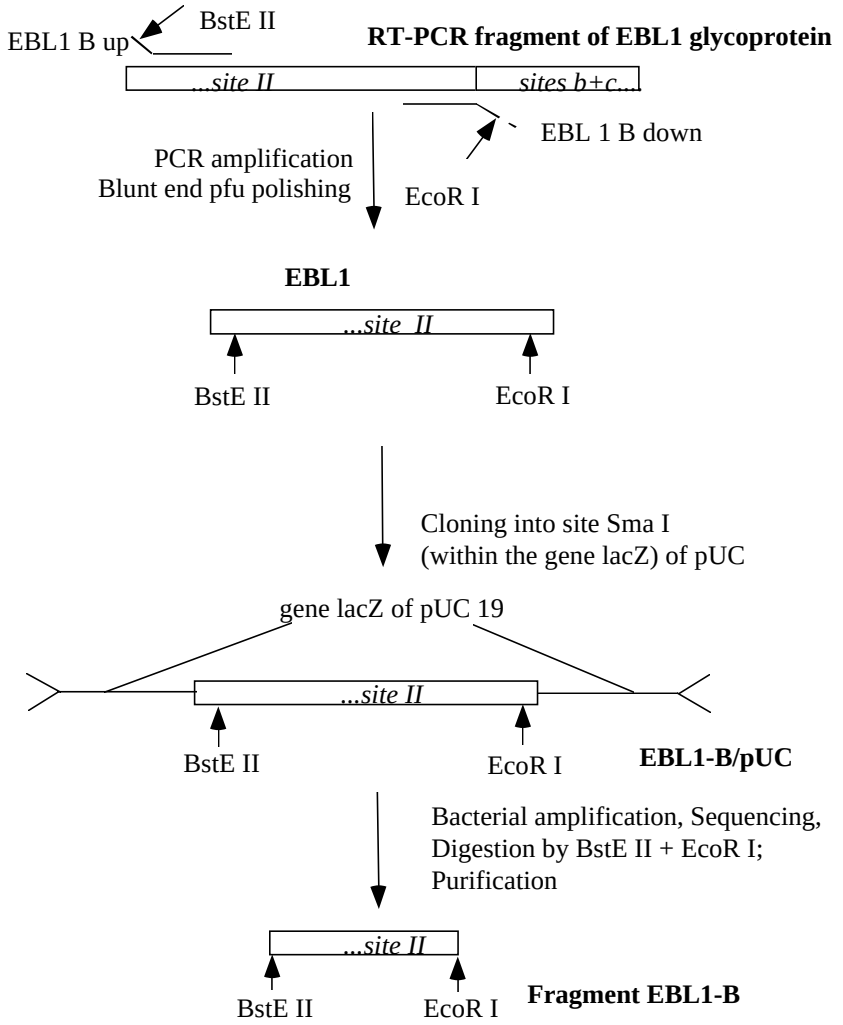


Figure III. 5.: Second step of cloning of EBL1/PV: Fragment EBL1-B.

Figure III. 6.

Cloning of pEBL1/PV

• *Summary (figure III. 4-6.):*

In order to produce a plasmid coding for a fusion protein EBL1/PV, e.g. containing the aminoterminal half of EBL1, and the carboxyterminal half of PV, an EBL1 fragment was inserted in pPVIII.

A RT-PCR transcript was available in the laboratory (H. Badrane), but it did not represent the complete EBL1 G. Therefore, in a first step an incomplete site II fragment was generated by PCR, using primers that introduced a BstE II and an EcoR I restriction site on the up and down strand respectively. This fragment was cloned into the restriction site Sma I of the pUC plasmid (figure III. 1; figure III. 5.). The obtained plasmid was amplified and sequenced. The required insert was then cut out by digestion by BstE II and EcoR I and purified (fragment EBL1-B).

The missing part of the site II region is provided by an oligonucleotide coding for an EcoR I restriction site, the missing sequence finishing with a BstE II restriction site followed by a little supplementary sequence and an EcoR I restriction site (adaptor EBL1-A). The BstE II restriction site has been obtained by a silent mutation. After insertion of this adaptor into the site EcoR I of pPVIII the plasmid is amplified and controlled by sequencing.

The little supplementary sequence is cut out by digestion with EcoR I and BstE II, thus resulting in the vector EBL1-A/PVIII. The fragment EBL1-B obtained before is cloned into this vector (figure III. 5.). The plasmid pEBL1/PV is controlled by sequencing, amplified, purified and tested *in vitro* and *in vivo*.

One has to note that the fusion G encoded by this plasmid varies slightly from a natural G: after the PV signal peptide **) and between the EBL1 and the PVIII half *) amino acids had to be introduced to obtain the necessary restriction sites.

• *Vector EBL1-A/PVIII (figure III. 4.):*

A solution containing 200 pmol of each oligonucleotide (oligo EBL1 - up, oligo EBL1 - down, appendix III. 5.) is frozen and vacuumdried during 1 hour. The DNA is resuspended in 10 µl 250 mM TrisHCl pH 7.7. 50 µl of PCR oil are added. The tube is incubated for 5 minutes at 96°C, cooled down to room temperature and put on ice (EBL1-A). pPVIII is digested by EcoR I. The restriction enzyme is inactivated by 10 minutes incubation at 65°C. Proteins are extracted with phenol/chloroform as described under "vector PV site III" (p. 56). DNA is precipitated with ammoniumactetate (vector PVIII). After ligation with EBL1-A, the DNA is precipitated with sodiumacetate, washed with 70% ethanol and resuspended in 15 µl H_2O. Competent bacteria are transformed and positive clones are identified by PCR, using the primers "pCIn 5" and "träger control".

figure III. 4.

figure III. 5.

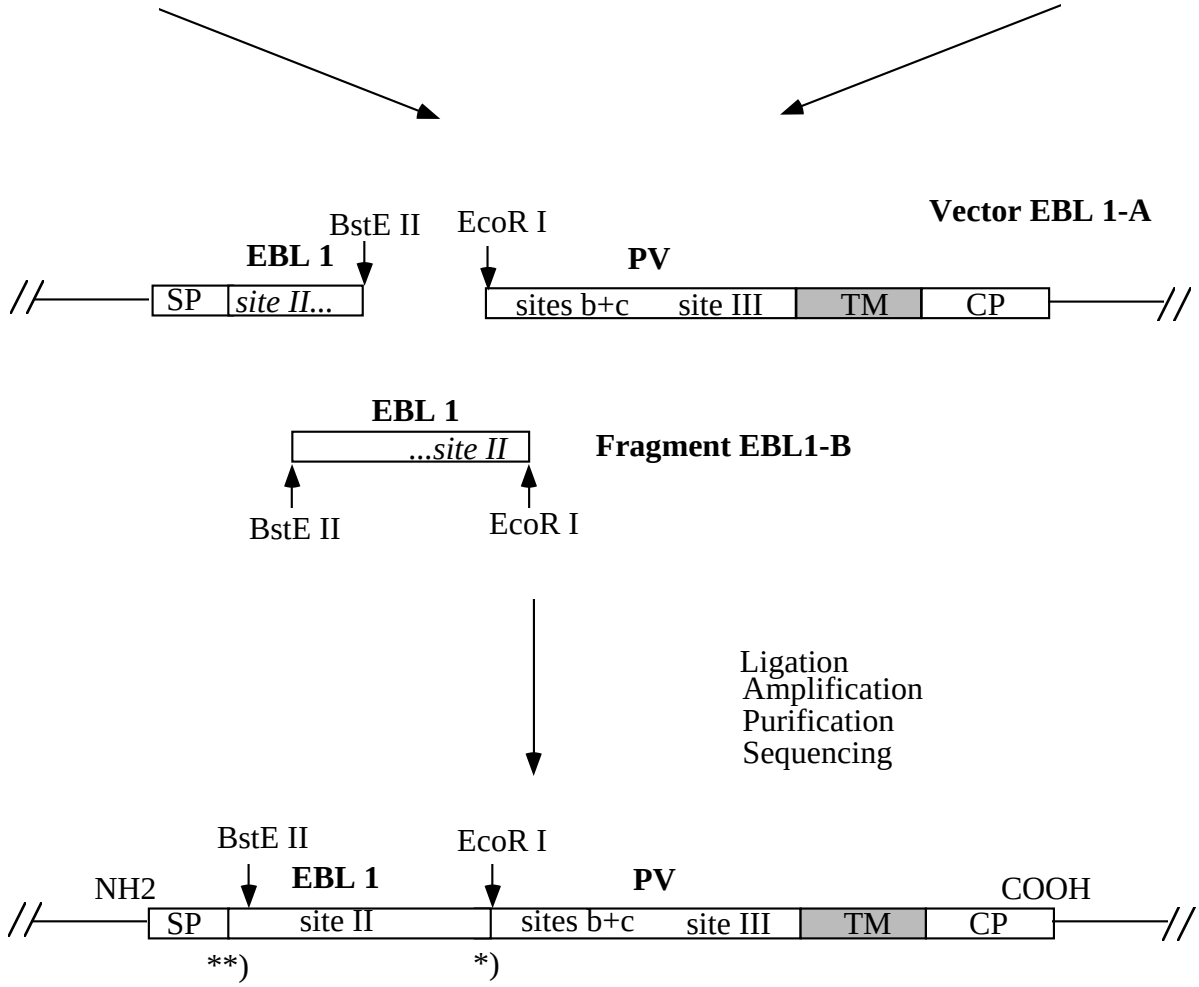


Figure III. 6.: Insertion of fragment EBL1-B into the vector EBL1-A.

• *Fragment EBL1-B (figure III. 5., p.59):*

A PCR amplification is realized with a RT-PCR product from EBL1 glycoprotein gene (H. Badrane) using the primers "EBL1B up" and "EBL1 down" (appendix III. 6.). The DNA is precipitated with sodiumacetate, resuspended in 100 ml H_2O and purified on acrylamide-bisacrylamide gel (EBL1-B).

As some DNA polymerases have been shown to generate 3-overhang extensions (Clark, 1988; Hu, 1993), the DNA fragment is treated with pfu polymerase (PCR Polishing Kit ,Stratagene) to increase the blunt-end cloning efficacy of the PCR fragment (Costa and Weiner, 1994). A final volume of 20 µl (2 µl 10X buffer Pfu (Stratagen), 2 µl dNTP 2,5 mM each (Stratagen), 5 u Pfu (Stratagen), 10 µl EBL1-B) is incubated for 30 minutes at 72°C.

pUC is digested with Sma I and the restriction enzyme is inactivated by 20 minutes incubation at 65°C. The digested vector pUC is linked to the fragment EBL1-B. The DNA is precipitated with sodiumacetate and resuspended in H_2O and

used for transformation of competent bacteria (EBL1-B/pUC). Positive clones are identified by bacterial lysis, amplified, purified and sequenced with the primer "M13/pUC" (see appendix III. 6.).

• *Ligation of EBL1-A and EBL1-B (figure III. 5.):*

One of each clone (EBL1-A/PVIII and EBL1-B/pUC) is amplified and purified by Qiagen. The two preparations are digested separately first with EcoR I then with BstEII. DNA is precipitated with ammoniumacetate. The vector EBL1-B is purified on 4% acrylamide-bisacrylamide gel and precipitated with ammoniumacetate. The two products are ligated and after transformation bacterial clones are screened by cracking. Positive clones are amplified, purified with the Wizzard Miniprep (Promega) and sequenced using the primers "pCIn 5", "EBL1II", "träger control", "GCH4", "GH7", "pCInR" (structure of EBL1/PV: appendix III. 1, sequence of open reading frame of pEBL1/PV: appendix III. 4., sequenced part of pEBL1/PV: appendix III. 7.).

A simplified view of the glycoprotein genes involved in plasmid preparation is shown in figure III. 7. The domains of the glycoproteins are placed in comparison to the viral glycoprotein of PV.

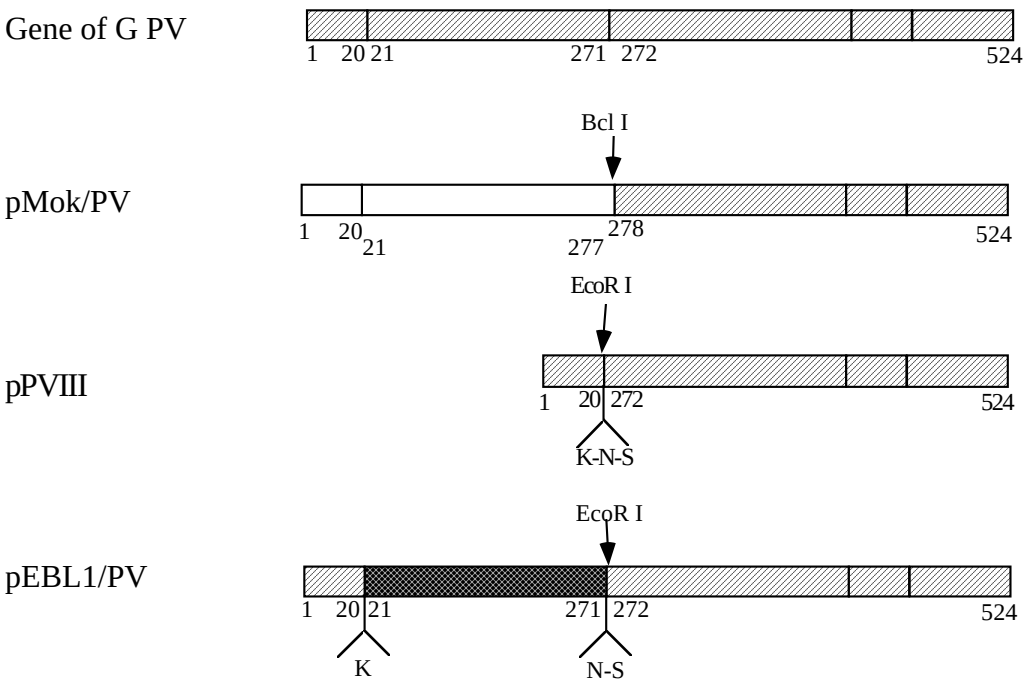


Figure III. 7.:
Simplified view of the glycoprotein genes used for plasmid preparation. The numbers refer to the aminoacid positions in the protein. Aminoacids that have been added for technical reasons are marked by ⅄ (sequences see appendix).

III. 9. Transient expression upon *in vitro* transfection of Neuro-2A cells

Wells of a 96 well cell culture plate (Falcon) are inoculated with $3x10^4$ Neuro-2a cells (in MEM 10% FCS) and incubated for 24 h at 37° C with 7.5% CO_2. The cells are washed with MEM without FCS and incubated without FCS for 1 hour at 37° C with 7.5% CO_2. After removal of the cell supernatant each well receives 50 µl of transfection solution as prepared: 0,1 µg of plasmid and 6 µl DOTAP transfection agent (Boehringer Mannheim) in sterile HEPES NaCl solution (20 mM HEPES - GibcoBRL-, 150 mM NaCl) incubated at room temperature for 15 min. The cells are incubated at 37° C with 7.5% CO_2 for 5 hours. 200 µl of MEM 2% FCS are added per well. The plate is incubated at 37° C with CO_2 for 24h. For further incubation the supernatant is removed and replaced by MEM containing antibiotics and 10% FCS. The plate is incubated at 37° C with CO_2 until glycoprotein expression is analysed by indirect immunofluorescence (see § III. 6.).

III. 10. Immunization of mice

Immunization with recombinant proteins

Recombinant and viral rabies proteins (Gs-Bac, G-Bac, N-Bac, RNP) are injected intraperitoneally in 0.5 ml PBS or NT. In some cases, the injection of N and RNP is accompanied by Complete Freund's Adjuvant (CFA).

DNA-based immunization

For *in vivo* testing of plasmids, mice were anesthetized with intraperitoneal injection of pentobarbital (0.08 mg/g body weight). Different quantities of plasmid were injected in 100 µl of PBS, half and half into the left and right calf of the leg or into the thigh. For DNA-based immunization with pPVIII, muscle degeneration was carried out before injection: 3.5 µg of cardiotoxin (Latoxan) were injected in 100 µl in the same manner as the plasmid four days before immunization of the mice.

For some experiments 10 international units (IU) of interleukin-2 (IL-2) were injected 30 min after the DNA solution at the same sites.

Bleeding of mice

Mice were bled by the retroorbital sinus. After coagulation of the blood the serum was collected, centrifuged at 2000 rpm for 5 min (Sigma 2K15) and the supernatant was incubated for 30 min at 55° C for elimination of complement activity. 1 mM of NaN_3 was added to each sample. The sera were stored at 4° C.

III. 11. Evaluation of the immune response

Antibody titration

VNA titration (RFFIT)

VNA were titrated using the rapid fluorescent focus inhibition test (RFFIT) (Smith et al., 1973) in a modified way (Perrin et al., 1986b). In a first step, the tested serum neutralizes a certain quantity of virus. In a second step, the non-neutralized virus is detected by its infection of cells. The intracellular RNP is detected by an anti-RNP antibody coupled to FITC (Sigma).

Several dilutions (in cell culture medium: MEM with antibiotics and 10% FCS) of the tested serum and of the international reference serum are distributed (100 µl per well) in a 96 well microplate (Falcon). 50 µl of a virus dilution (supernatant of infected BHK cells) that infects 80% of the cells are added to each well. The microplates are incubated at 37° C with 7.5% CO2 for 1 hour. 50 µl of cell suspension (BHK-21 C13) containing $5x10^4$ cells are added to each well. The plates are incubated under the same conditions for 24 h in the case of CVS or PV and 48h the case of EBL1 and Mokola. The supernatant is removed carefully, the cell layer is washed with PBS Ca^{2+}/Mg^{2+} and fixed at 4° C in 80% chilled acetone. After elimination and evaporation of the acetone, the anti-RNP antibody, coupled to FITC is added (50 µl per well) and incubated for 1 hour at 37° C. The plates are washed with PBS.

The titre of VNA is determined by comparing the dilution of serum that induced 50% reduction of fluorescent foci to the dilution of reference serum that had the same consequence. For genotype 1 the result is directly expressed in international units per ml (IU/ml). For other genotypes, a serum dilution inducing a 50% inhibition of the fluorescent focus rate is considered to contain the same VNA titre as the genotype 1 under identical conditions. VNA titres may as well be expressed as the reciprocal serum dilution that induces 50% reduction of virus infectivity.

Titration of antibody by ELISA

The antibody titres against rabies antigens are determined by ELISA as described (Perrin et al., 1986a). The tested sera are incubated in sensibilized microplates (200 µl of antigen 10 µg/ml per well). For titration of anti-RNP antibodies the wells are coated with RNP, for titration of anti-virus antibodies the wells are coated with inactivated purified rabies virus (IPRV). The serum antibodies fixed on the antigen are detected by an anti-IgG-mouse serum coupled to peroxydase (Nordic). The coating of the plates, titration and detection are carried out as in the general paragraph about ELISA (see § III. 8.). The results are expressed in comparison to the international reference serum (IU/ml).

T helper cell response: IL-2 production by splenocytes upon *in vitro* stimulation

Splenocytes of immunized mice are stimulated with different antigens to produce IL-2. The biological activity of IL-2 is quantified *in vitro* following a bioassay that measures proliferation of indicator cells (CTLL) which are IL-2 dependent. The quantification of IL-2 is carried out as described by Gillis (Gillis et al., 1978), adapted by Oth et coll. (Oth et al., 1987) and modified by Joffret et coll. (Joffret et al., 1991).

The spleens of mice are crushed in RPMI medium between two roughened microscopic slides. The splenocytes are centrifuged (900 g, 6 min) and the pellet is resuspended by agitation. The red blood cells are eliminated by osmotic shock: 1.5 ml of chilled H_2O is added to the cell pellet and agitated for 7 seconds. The lysis is stopped by addition of 10 ml RPMI medium. The splenocytes are centrifuged as before and the pellet is washed twice in RPMI and resuspended in complete RPMI. The cells are counted and adjusted to a concentration of $6x10^6$ cells/ml.

For stimulation of splenocytes, medium and different antigens are distributed in a 24 well plate (Falcon). The quantity of the different antigens distribruted per well in 100 µl complete RPMI medium is as follows: 0.1 µg IPRV PV, 0.5 µg IPRV EBL1, 0.5 µg of purified G PV, 0.5 µg of purified RNP PV, 5 µg of Concanavalin A (ConA; Miles Yeda, Kankakee, IL) for unspecific T cell stimulation. The cells are incubated at 37° C with 7.5% CO_2 for 24 h. The supernatant is collected and centrifuged. After centrifugation the supernatant is stored at -20° C.

The CTLL cells are prepared in order to be in the exponential growth phase at the day of IL-2 quantification: $2x10^6$ cells are cultivated with rat IL-2 in 10 ml of complete RPMI medium for 24 h. At the day of quantification the cells are washed twice with RPMI and resuspended in 5 ml of complete RPMI without sodium pyruvate and non-essential amino acids. After incubation at 37° C with 7.5% CO_2 for 1 h 30 the cells are washed again twice in order to eliminate all traces of IL-2 and subsequently resuspended in complete RPMI medium with antibiotics. The reference IL-2 (recombinant mouse IL-2) dilutions and the supernatants of stimulated splenocytes are distributed by triplicates (100 µl per well, in the case of ConA supernatant diluted 1/1 with complete RPMI medium) in a 96 well microplate (Falcon). 50 µl of CTLL cell suspension (4x105 cells/ml) are added per well and the cells are incubated at 37° C with 7.5% CO_2 for 24 h. The cells are labeled with radioactivity by adding 50 µl containing 1 µCi ^3H thymidine (NEN Dupont de Nemours, Paris) per well and incubation of 5 h under the same conditions. After cell lysis the radioactive DNA is collected on filters using the cell collector (Skatron, Norway). The radioactivity is measured by counting in scintillation liquid (Amersham) in counts per minute (cpm). The results are calculated by comparison to the graph obtained with the reference IL-2 and expressed in units (U)/ml (Gillis et al., 1978). Alternatively, results may be expressed as cpm.

RESULTS

Injection	VNA titre (IU/ml)
Gs-Bac	3.2
G-Bac	0.3 (± 0.1)
G-Bac in liposomes	4 (± 2.4)
IPRV	22.6 (± 15.4)

Table IV. 1.: Immunogenicity of the recombinant G protein from extracellular (Gs-Bac) or intracellular (G-Bac) origin: virus neutralizing antibody production. Mice have been injected intraperitoneally on day 0 and day 7 with 1 µg of recombinant G. For reference, mice received one injection of 1 µg IPRV. The sera were controlled on day 14. The data shown indicate the VNA titre (IU/ml) against CVS.

IV. RESULTS

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IV. 1. Improvement of recombinant rabies glycoprotein immunogenicity by association with recombinant nucleoprotein

As described in the introduction (see § I. 1. 7. b.), the role of the nucleoprotein (N) in anti-rabies immunity has not yet been fully clarified. In order to determine whether the addition of nucleoprotein to a glycoprotein (G) immunization would improve its immunogenicity we have produced both proteins in the baculovirus expression system in order to assure N preparations that are free of G contaminations and vice versa.

IV. 1. 1. Immunological properties of recombinant glycoprotein

Before investigating the influence of recombinant nucleoprotein on the immunogenicity of recombinant glycoprotein, G has been tested for its immunological properties and for their dependence on the origin of recombinant G, on its conservation and on its presentation.

Recombinant G of two origins has been differentiated: recombinant G in the cell extract (G-Bac) and recombinant G that has been secreted into the cell supernatant (Gs-Bac). Both forms are immunologically active as they are able to induce virus neutralizing antibodies (VNA). The secreted form of G is more immunogenic than the cell extract (table IV. 1.).

When recombinant G is stored at 4° C, a considerable loss of antigenicity (as estimated by ELISA) has been observed in spite of the presence of anti-proteases (data not shown). For better conservation G has been freeze-dried. Furthermore freeze-drying was necessary to obtain a concentrated product for later encapsulation with liposomes in association with N (see § IV. 1. 3. a.). The process of lyophilization deteriorates the immunogenicity (as controlled by VNA induction) of Gs-Bac, whereas G-Bac maintains its immunological properties (data not shown), possibly because of a stabilization by contaminating insect cell proteins. As Gs-Bac could not be concentrated, liposomes were only prepared with G-Bac. Their immunogenicity was by far superior to the immunogenicity of G-Bac alone. Nevertheless, the level of VNA induced by recombinant G stayed in all cases far below the VNA titre obtained after one injection with the same amount of inactivated purified rabies virus (IPRV, table IV. 1.).

Thus, two different forms of immunologically active G have been obtained with the baculovirus expression system: Gs-Bac that is a potent immunogen and

* The individual data of the results shown in this chapter are listed in appendix IV.

Injection	**Th cell response (T cell growth, cpm) after stimulation with**			**Anti-RNP antibodies (U/ml)**
	medium	*RNP*	*IPRV*	
RNP	3865 (± 2647)	75876 (± 3106)	38289.5 (± 7624.5)	36.8
N-Bac	3463.5 (2199.5)	74158 *	46287 *	32.8

* The second mouse injected with N-Bac did not respond, the result was not included in this table (see appendix IV).

Table IV. 2.: Immunogenicity of the N protein: Th cell response and antibody production. Mice have been injected intraperitoneally with 5 µg of RNP or N-Bac. The Th cell response was evaluated at day 7 by the IL-2 production (counts per minute, cpm) upon *in vitro* stimulation of the splenocytes with RNP and IPRV. The IL-2 produced after stimulation was assayed as described (see § III. 11). Anti-RNP antibodies were measured on day 21 with the ELISA test.

Injection			**Protection**
First injection (amount)	Second injection (amount)	Adjuvant (CFA)	
N-Bac (10 µg)	-	yes	0%
N-Bac (15 µg)	RNP (5 µg)	no	30%
N-Bac (15 µg)	RNP (5 µg)	yes	30%
RNP (10 µg)	-	yes	31%
RNP (5 µg)	RNP (5 µg)	no	80%
RNP (5 µg)	RNP (5 µg)	yes	100%

Table IV. 3.: Protection against intramuscular challenge after injection of different quantities of RNP and N-Bac, with or without boost with RNP on day 13. The antigen was injected with or without Complete Freund's Adjuvans (CFA). The given percentage indicates the survival after challenge with CVS on day 20.

G-Bac that is poorly immunogenic but that can be considerably improved by encapsulation with liposomes.

IV. 1. 2. Immunological properties of recombinant N protein

The immunological properties of recombinant N protein (N-Bac) have been compared to purified viral ribonucleoprotein complex (RNP). It is important to note that sufficient purification of RNP is only obtained after purification on CsCl gradient. RNP purified on saccharose gradient induces VNA (data not shown), indicating a contamination with viral G.

Recombinant N has been compared with viral RNP concerning the induction of a T helper (Th) cell response (appraised according to the interleukin-2, IL-2, production by activated Th cells) and the induction of anti-RNP antibodies. N-Bac is able to induce a Th cell response that can be stimulated *in vitro* by both, viral RNP and IPRV, in a similar manner as with the Th cell response obtained with viral RNP. N-Bac could also stimulate anti-RNP-antibody production in a similar level as RNP (table IV. 2.). Both N presentations have been tested for their capacity to protect against an intramuscular challenge. A single injection of N-Bac does not induce any protection at all, whereas a partial protection has been observed with RNP. The protection by N-Bac can be improved by injecting higher quantities and by boosting with RNP. Nevertheless, the protection achieved by N-Bac stays far inferior to the protection obtained by RNP in the same conditions (table IV. 3).

Recombinant N is therefore equivalent to RNP concerning induction of both, Th cell response and anti-RNP antibodies. As N-Bac will be associated to G for its properties as a Th cell inductor, its weak protective activity can be neglected.

IV. 1. 3. Influence of the association of recombinant nucleoprotein to recombinant glycoprotein on the immunological properties of the glycoprotein

The association of recombinant N to recombinant G has been tested according to two protocols with two injections: 1) two injections of a G+N protein mixture (in order to test a possible adjuvant effect of the N protein); 2) a first injection with recombinant N followed by a boost with a G+N protein mixture (in order to test a possible priming effect of the N protein).

IV. 1. 3. a. Adjuvant effect of recombinant N protein

Both recombinant proteins, G-Bac and Gs-Bac, have been tested in combination with N-Bac.

For investigation of the immunogenicity of a Gs-Bac+N association we have chosen homogenization by ultrasonication (60 Hz, twice 20 seconds) as Gs-Bac could not be concentrated for encapsulation by liposomes. When N-Bac was mixed with

Gs-Bac without further treatment (Gs-Bac+N-Bac) the immunogenicity of Gs-Bac was not significantly enhanced. On the contrary, when the same mixture was homogenized by ultrasonic treatment ((Gs-Bac+N-Bac)US) the immunogenicity of Gs-Bac was clearly improved. The effect of N-Bac in the homogenized mixture was even more evident when compared to the immunogenicity of ultrasonicated Gs-Bac on its own ((Gs-Bac)US), which had obviously been deteriorated by the effect of ultrasonication. The effect of ultrasounds on a Gs-Bac+N-Bac mixture has been reproduced.

Concerning G-Bac the influence of the association of different amounts of N-Bac was investigated in two types of experiments using either a simple mixture of both proteins or the same mixture encapsulated by liposomes. As described in figure IV. 5. the adjuvant effect was most obvious after association of 0.1 µg of N-Bac to G-Bac, whatever the form of association. A higher or lower amount of N-Bac induced less increase of VNA. In any case, the VNA titres obtained after two injections of recombinant proteins were far inferior the titres induced by only one immunization with IPRV.

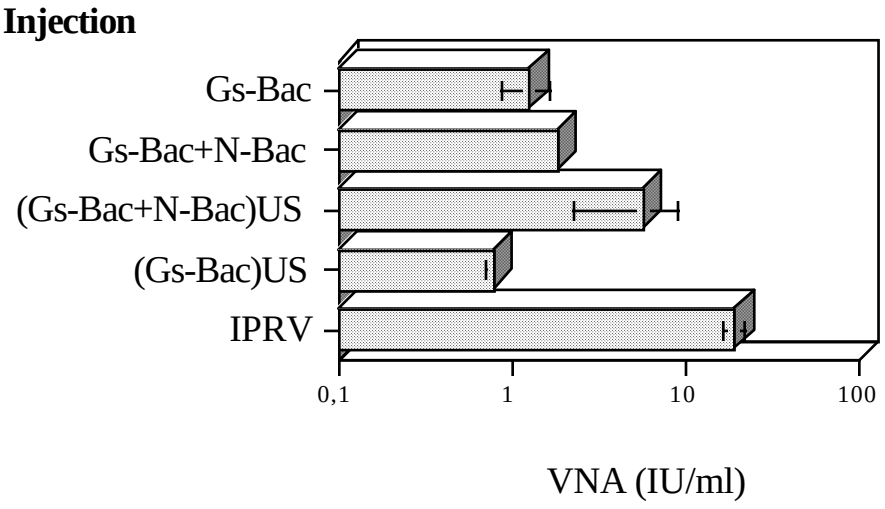


Figure IV. 4.: Adjuvant effect of recombinant N on the immunogenicity of recombinant G. Mice have been injected twice (day 0 and day 7) with 0.4 µg Gs-Bac and 1 µg N-Bac. The preparations were injected either as a simple mixture (Gs-Bac or Gs-Bac+N-Bac) or after homogenization with ultrasounds ((Gs-Bac)US or (Gs-Bac+N-Bac)US. Control mice received one injection of 1 µg IPRV. The sera were tested for VNA (IU/ml) against CVS on day 20.

Thus with both forms of recombinant G an adjuvant effect of N-Bac has been observed. In the case of Gs-Bac, this effect depended on a previous homogenization of the protein preparation. Despite of the enhancing effect of liposomes on G-Bac no difference in immunogenicity was noticed whether the mixture of G-Bac+N-Bac was encapsulated with liposomes or not. Regardless of the adjuvant effect of N, even the addition of N to two injections with G did not rise the antibody production to a similar level as one injection of IPRV.

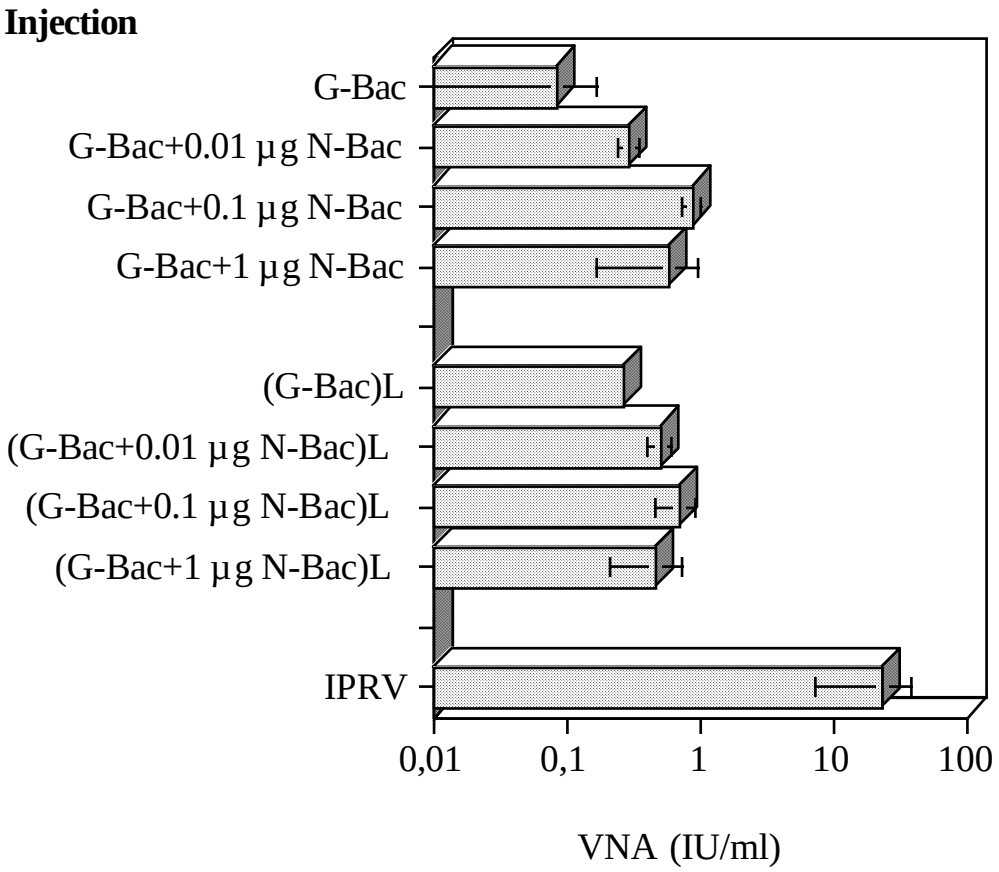


Figure IV. 5.: Adjuvant effect of recombinant N on recombinant G. Mice have been injected twice (day 0 and day 7) intraperitoneally with 0.1 µg G-Bac and different quantities of N-Bac (0.01, 0.1 or 1 µg). The preparations were injected either without further treatment (G-Bac or G-Bac+N-Bac) or after encapsulation with liposomes ((G-Bac)L or (G-Bac+N-Bac)L). Control mice received one injection of 1 µg IPRV. The sera were tested on day 14 for VNA (IU/ml) against CVS.

IV. 1. 3. b. Priming of N-Bac for a mixture of G-Bac+N-Bac

In order to determine whether N-Bac would be able to prime for a following injection of a combination of G-Bac and N-Bac, the VNA titres of non-primed animals that had received a mixture of G-Bac and N-Bac were compared to the VNA titres of animals that had been primed with N-Bac before injection of G-Bac+N-Bac. No detectable titres of VNA have been measured no matter whether the animals had been primed or whether for the boost G-Bac+N-Bac were encapsulated with liposomes or not (data not shown).

Thus no priming by N-Bac for a G-Bac+N-Bac mixture could be demonstrated.

IV. 1. 3. c. VNA-induction against European bat lyssavirus 1 after injection of Gs-Bac in association with N-Bac

In order to determine whether recombinant G protein could be used to induce protection against European bat lyssavirus (EBL1), glycoprotein preparations were tested for their capability to induce neutralizing antibodies against EBL1. It has been shown that the association of N-Bac to Gs-Bac in a homogenized mixture lead to a considerable increase of VNA against the challenge virus strain (CVS). However, no similar increase is observed concerning EBL1 (table IV. 6.).

Consequently, even though the association of recombinant N and G can induce a satisfactory level of VNA against the genotype 1 (figure IV. 4.), the production of VNA against EBL1 is only very weak. For immunization against European lyssaviruses, a new approach has therefore been chosen.

Injection	**VNA titre against**		**Ratio ($x10^{-2}$)**
	CVS	**EBL1**	**EBL1:CVS**
Gs-Bac	200	40	20
Gs-Bac/N-Bac	200	28	14
(Gs-Bac+N-Bac)US	1100	95	8.6

Table IV. 6: Cross-reactivity after injection of different preparations containing recombinant G and N proteins. The sera of mice that have been injected as in figure IV. 4 and figure IV. 5. have been pooled and VNA against CVS and EBL1 have been titrated. The titre indicated corresponds to the reciprocal serum dilution that induced 50% reduction of virus infectivity.

IV. 2. Improvement of rabies DNA-based immunization against European bat lyssaviruses by chimeric genes

During previous experiments in the Lyssavirus laboratory, a DNA-based immunization with Pasteur virus (PV) glycoprotein has induced protection against genotype 1. In the aim to broaden the spectrum of protection of vaccination towards the African genotypes a plasmid has been constructed that coded for a protein chimera, containing the carboxyterminal part of PV and the aminoterminal part of Mokola (Mok), the plasmid pMok/PV. This chimera has been able to induce a potent immune response with protective titres of VNA against all genotypes but Duvenhage and EBL1 (Bahloul et al., 1997). In this study a similar plasmid pEBL1/PV, coding for a hybrid protein EBL1/PV has been constructed and tested for its immunological characteristics and for its capability to achieve protection against PV and EBL1.

IV. 2. 1. Induction of virus neutralizing antibodies by pEBL1/PV

The injection of the chimeric plasmid pEBL1/PV induced an early and high VNA production against both, EBL1 and PV (figure IV. 7.). The VNA titres against PV were superior to the limit of protection that has been shown to be between 1.0 and 1.5 international units (IU) per ml (P. Perrin, personal communication).

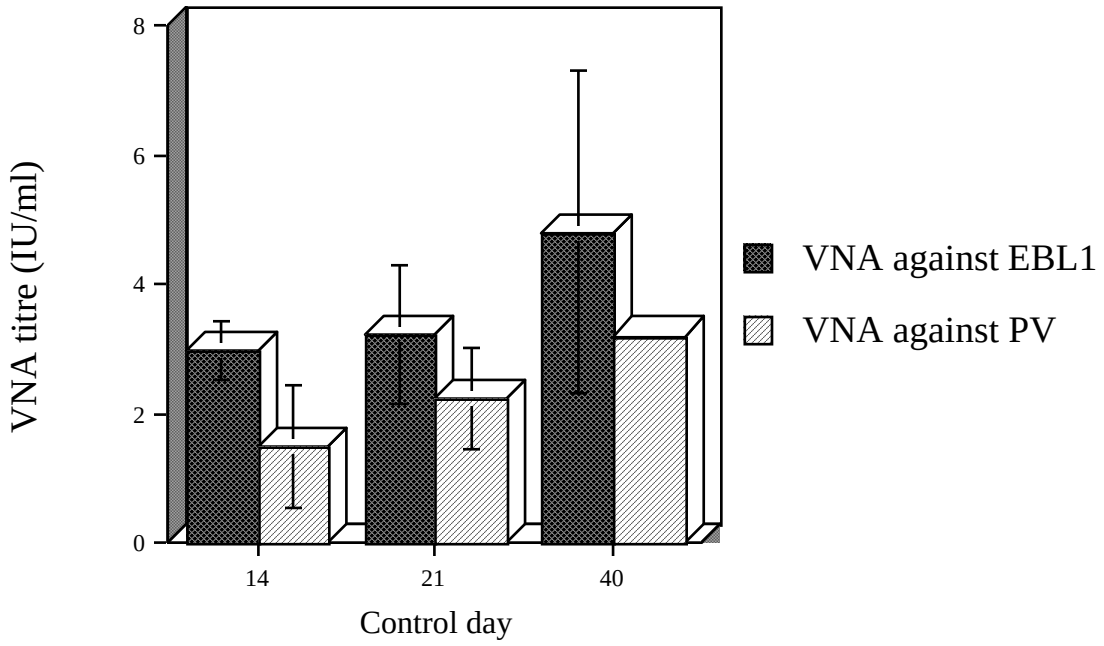


Figure IV. 7.: Kinetics of VNA (IU/ml) production against both, EBL1 and PV viruses after intramuscular injection of mice with 40 µg pEBL1/PV.

However, whatever the day of control, the VNA titres against EBL1 were always higher than those against PV. Antibody production continued to rise until the last control point at 40 days.

IV. 2. 2. T-helper cell response

As the Th cell response is very important in anti-rabies immunity, the chimeric plasmid pEBL1/PV has been tested for its capability to induce Th cells that produced IL-2 after *in vitro* stimulation by lyssavirus antigens. In fact, the Th cell response was very high concerning both EBL1 and PV. IL-2 production was similar to the levels obtained after immunization with the positive control, pPV/PV (figure IV. 8.). The threshold of detection of IL-2 was at 0.08 U/ml. As the empty plasmid vector, pCI-neo, did not induce any Th cell response, the obtained results with pEBL1/PV and pPV/PV represent a specific response.

Consequently, the chimeric plasmid pEBL1/PV is able to induce a Th cell response which can be stimulated by both parental antigens.

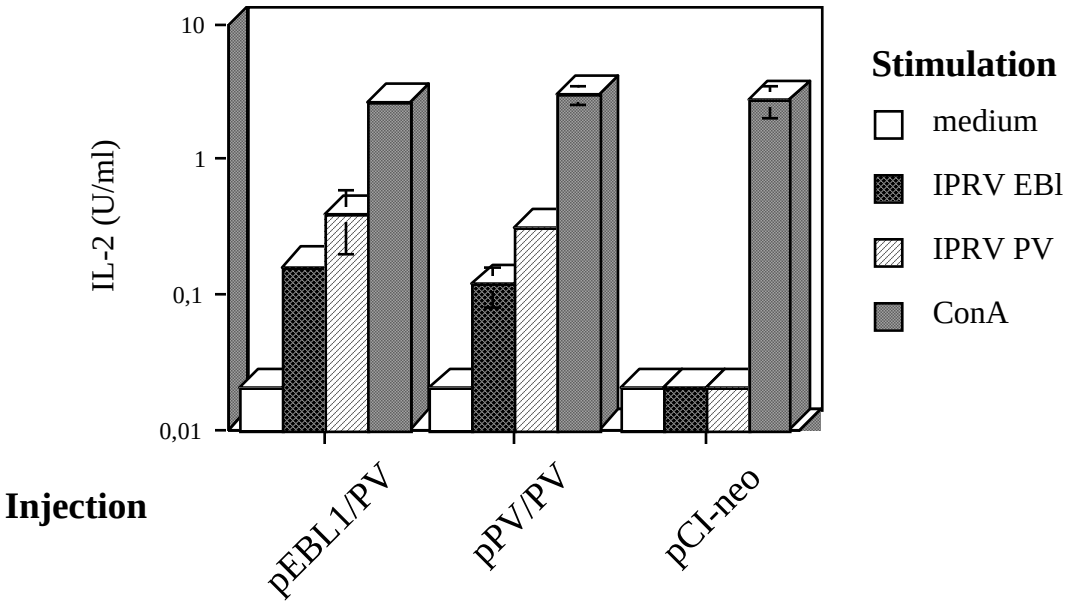


Figure IV. 8.: IL-2 (U/ml) production after*in vitro* stimulation of splenocytes from mice that have been injected with 40 µg of pEBL1/PV, pPV/PV or pCI-neo. The response to stimulation with IPRV (EBL1 and PV) was mesured 14 days after injection.

IV. 2. 3. Comparison of immunogenicity of two lyssavirus glycoprotein gene products (pEBL1/PV and pPV/PV): VNA induction and protective activity

In order to determine if the chimeric plasmid pEBL1/PV can be used as "vaccine" against viruses from both genotypes, 1 and 5, this construction has been tested against the two virus strains in comparison with the homologous construction pPV/PV. VNA production and protection has been investigated.

DNA-based immunization with pEBL1/PV induced by day 14 a considerable VNA titre against EBL1, whereas the VNA titre against PV was half as high. At the same time pPV/PV had induced a higher VNA titre against PV than against EBL1. On day 21 all VNA titres are higher than on day 14 but the difference between the two constructions and viruses tested stayed the same (table IV. 9. a.).

Injection	VNA (UI/ml) on day 14 against		VNA (UI/ml) on day 21 against	
	EBL1	PV	EBL1	PV
pPV/PV	0.8 (± 0)	1.2 (± 0.4)	1.8 (± 1.4)	3.3 (± 3.1)
pEBL1/PV	1.2 (± 0.4)	0.6 (± 0.2)	3.6 (± 2.8)	1.2 (± 0.4)
pCI-neo	< 0.4 (± 0)	< 0.2 (± 0)	< 0.4 (± 0)	< 0.2 (± 0)

Table IV. 9. a.: Comparison of VNA titres against PV and EBL1 at day 14 and day 21 after injection of 40 µg pEBL1/PV, pPV/PV or pCI-neo (negative control).

The protective activity of pEBL1/PV and pPV/PV against intracerebral challenge has been compared. pEBL1/PV induced the same level of protection against EBL1 and CVS, while pPV/PV did not induce any protection against EBL1 and only very partial protection against CVS (table IV. 9. b.). The low level of protection of pPV/PV against CVS is surprising (Bahloul et al., 1997). Actually, our experiment has been carried through with Balb/c mice and a control of the protective activity of pPV/PV on OF1 mice yielded 86% protection against CVS indicating that the conditions of challenge of the Balb/c mice were particularly stringent.

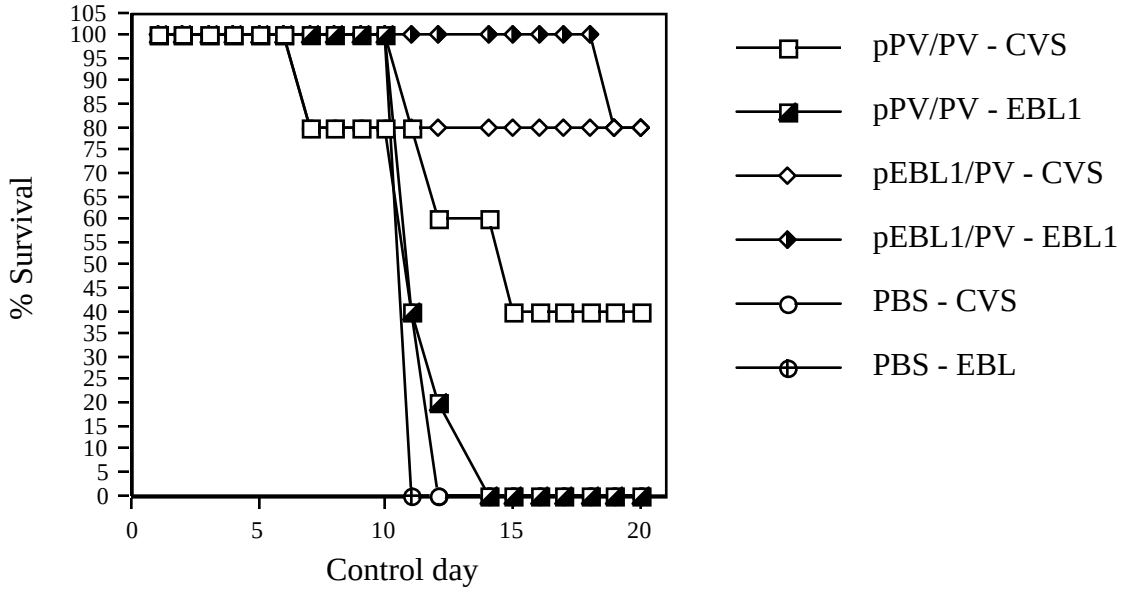


Figure IV. 9. b.: Comparison of the protective activity of pEBL1/PV and pPV/PV towards an intracerebral challenge with EBL1 and CVS. Mice were injected with 50 µg pEBL1/PV or pPV/PV or with phosphate buffer saline (PBS; control animals) and challenged with EBL1 and CVS after 21 days (identification of the curves: injection - challenge). The results indicate the percentage of surviving animals.

We have shown that the chimeric plasmid pEBL1/PV is able to induce a high Th cell response and high titres of VNA against EBL1 and PV. The protection achieved by this construction against EBL1 is superior to the protection achieved by pPV/PV, while maintaining protection against CVS: only pEBL1/PV was able to induce protection against intracerebral challenge against both EBL1 and CVS. DNA-based immunization would therefore be a possible vaccine against both, genotype 1 and 5.

IV. 3. Structure and immunological properties of the rabies glycoprotein:

Investigation by cell transfection and DNA-based immunization with various lyssavirus glycoprotein genes

Plasmid technology is a powerful tool to study *in vitro* expression of antigens as well as their immunological properties as appraised by DNA-based immunization. For a preliminary study of the relation between *in vitro* expression and immunogenicity of the lyssavirus glycoproteins, different glycoprotein constructions (figure IV. 10.) have been tested for the quality of the expression of their

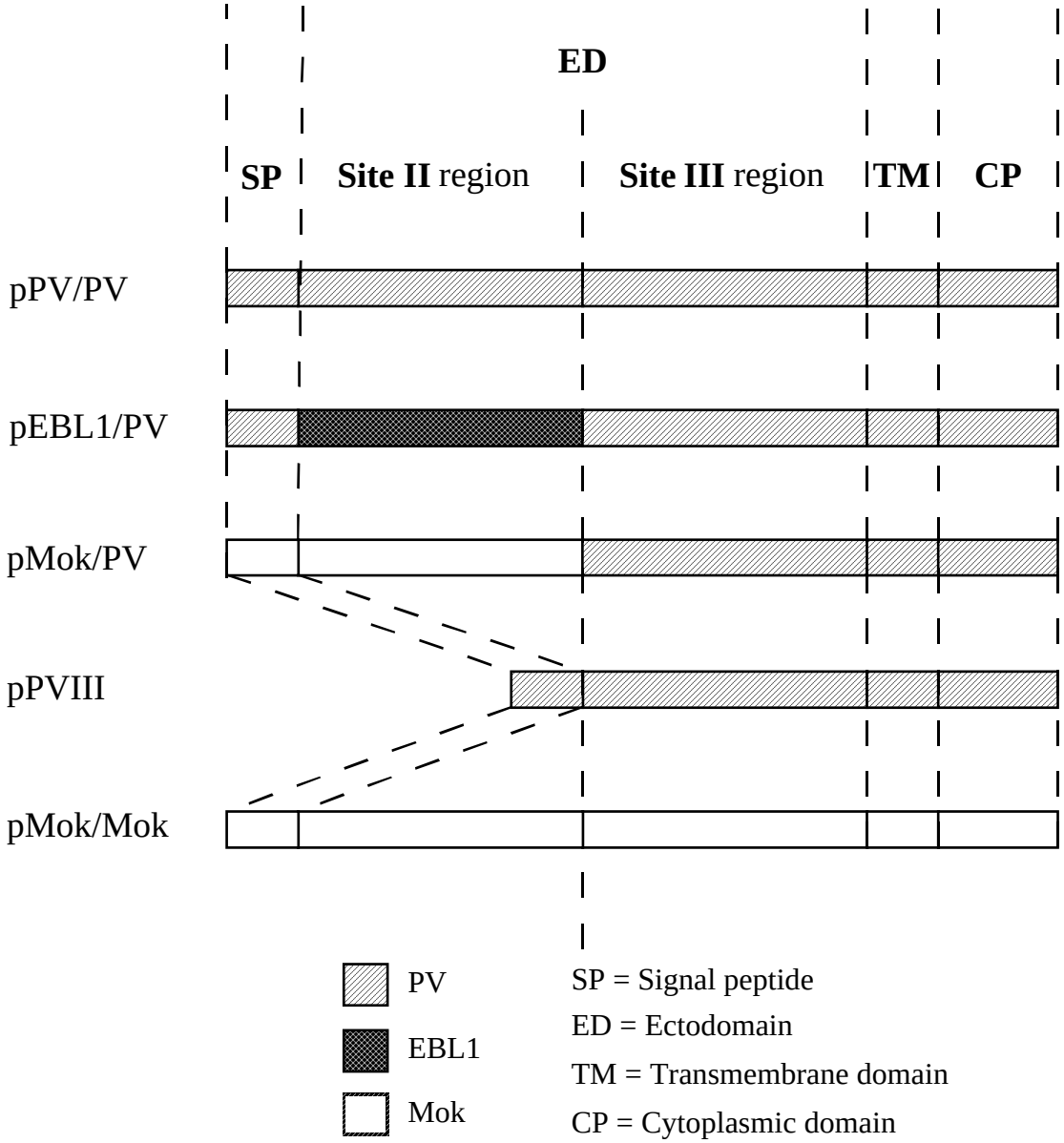


Figure IV. 10.: Schematic view of the linear structure of different lyssavirus glycoprotein constructions that have been tested *in vivo* and *in vitro* (see also figure III. 7.)

Transfected plasmid	Immunofluorescent staining with						Cell morphology	
	PVE12	D1	6-B1	anti-G PV	anti-G Mok	anti-EBL1	Foci	Single cells
pPV/PV	30% +	30% +	1 % +	30% 1% +	20% +/-	20-30% +/-	++	±
pEBL1/PV	0	30-40% +	10% +	50% 5% +	-	30-40% +	+++	+
pMok/PV	0	10% +	1-5% +/-	10% F +	10-20% +	-	++	±
pPVIII	0	0	5-10% +	10-20% +	0	0	0	+
pMok/Mok	0	0	0	10% 1-2% +/-	30% 1% +	-	++	±

+/- = weak fluorescence
+ = normal fluorescence
- = not tested

Table IV. 11.: Percentage and appearance of positive Neuro-2a cells after immunofluorescent staining with different monoclonal (mono) and polyclonal (poly) antibodies, 96 hours after transfection with different plasmids. The results shown concern fixed cells, no significant difference in percentage and quality of fluorescence have been observed if the cells were not fixed before immunofluorescent staining. Monoclonal antibodies: PVE12 (conformational site II region), D1 (conformational site III region), 6-B1 (denatured site III region); polyclonal antibodies: anti-G PV, anti-Mok PV, anti-EBL1.

glycoprotein products in neuroblastoma cells and to induce immune responses in mice after intramuscular injection.

IV. 3. 1. Structure: *In vitro* expression of various lyssavirus glycoproteins and antigenic analysis

The expression after *in vitro* transfection of Neuro-2a cells with plasmids coding for homogeneous, heterogenous and truncated lyssavirus glycoproteins has been analysed by indirect immunofluorescence (table IV. 11. and figure IV. 12.). In order to simplify the description of the results, the aminoterminal half of the glycoprotein will be called "site II region" and the carboxyterminal half "site III region".

For antigen staining two types of antibodies were available: 1) polyclonal (poly) antibodies against the G PV or G Mok or against EBL1 IPRV and 2) monoclonal (mono) antibodies against the conformational sites II (PVE12) and III (D1) of G PV and against the denatured site III region of G PV (6-B1). For negative control, Neuro-2a cells have been transfected with the empty plasmid vector (pCI-neo). No fluorescence has been observed after immunostaining, no matter, which antibody was used (a part of the data is shown in figure IV. 12. 1-4. f.).

The conformational antigenic site II of the PV glycoprotein has been recognized by the antibody PVE12 on pPV/PV transfected cells as well as on PV infected cells indicating a correct folding of G on the transfected cells. The correctly folded antigenic site III has been evidenced on pPV/PV, pEBL1/PV (figure IV. 12. 3. a. and b.) and on pMok/PV transfected cells. Again, the result obtained was similar to the one on PV infected cells (figure IV. 12. 3. d.). Mainly pPVIII (figure IV. 12. 4. c.) transfected cells were stained by the antibody 6-B1, whereas no fixation of 6-B1 on PV infected cells has been observed (figure IV. 12. 4. d.).

When transfected cells are stained with polyclonal antibodies the results are less conclusive as a fixation does not give any information about the state of the expressed protein. Basically, all cells that have been transfected with a plasmid containing a PV, EBL1 or Mok fragment have been recognized by the polyclonal antibodies against PV, EBL1 or Mok, respectively. In some cases cross-reactivity has been observed (figure IV. 12. 1. and 2. a., b., d. and e.).

Figure IV. 12.(in front): Immunofluorescent staining of Neuro-2a cell after transfection with different plasmids or infection with different viruses.

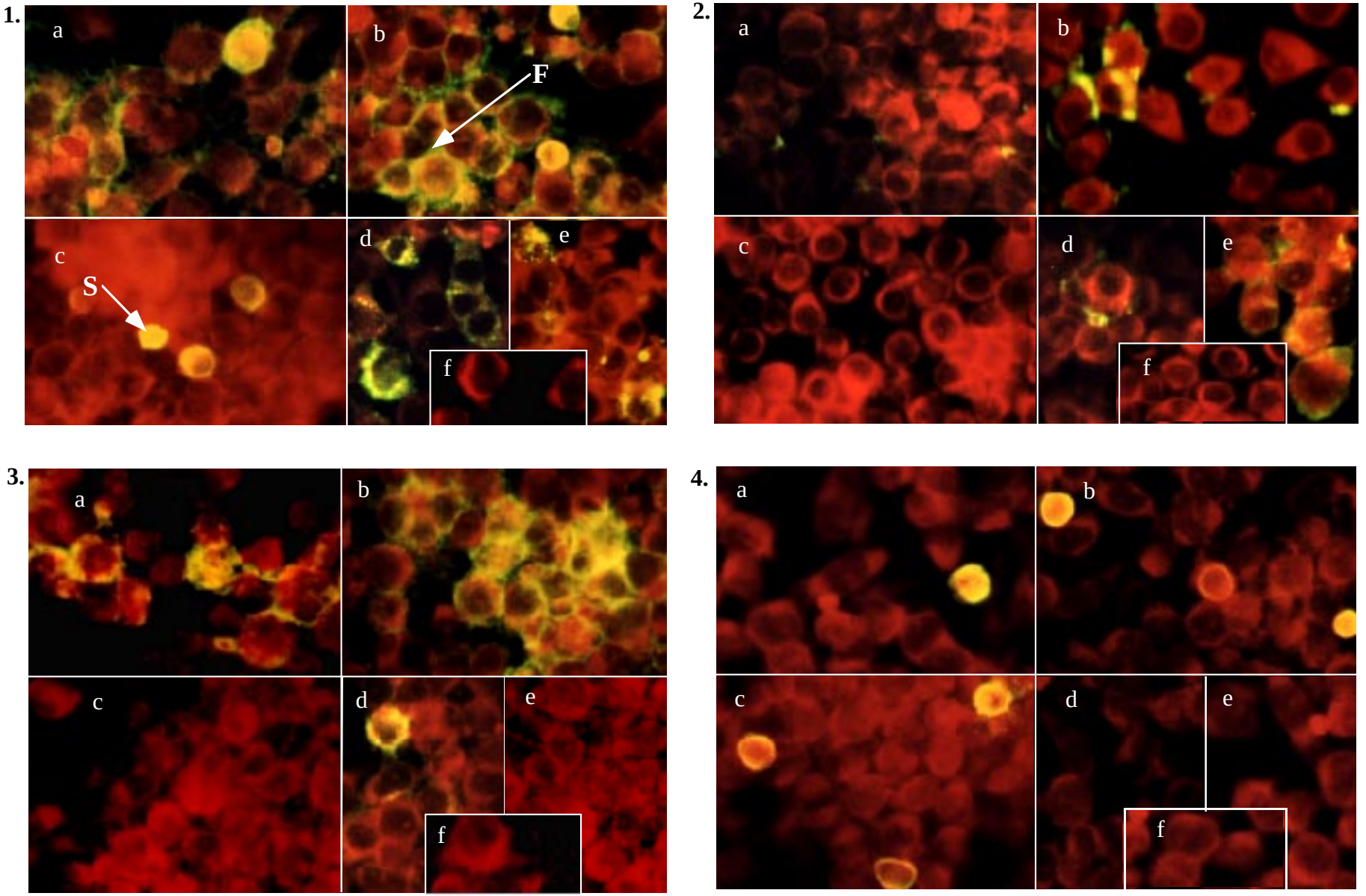
Transfection of Neuro-2a cells with
a. pPV/PV
b. pEBL1/PV
c. pPVIII
f. pCI-neo (empty vector)

Infection of Neuro-2a cells with
d. PV virus
e. EBL1 virus

F = focus, S = single cell

Immunostaining with
1. poly. anti-G PV
2. poly. anti-EBL1
3. mono. D1
(conformational site III region PV)
4. mono. 6-B1
(denatured site III region PV)

Figure IV. 12.



In order to determine whether the expression of an altered glycoprotein (recognition by 6-B1) was an early or a late event in the expression by transfected cells, a kinetic analysis over 144 hours has been carried out with cells transfected with pEBL1/PV, pPVIII or pPV/PV (figure IV. 13., for detailed results see appendix IV). Whatever the day of control, pEBL1/PV transfected cells were stained with the anti-G PV (figure IV. 13. a.) and the monoclonal D1 antibody (data not shown), indicating that the chimeric glycoprotein is mainly expressed with a correct folding. However, as shown in figure IV. 13. b., an increasing staining with 6-B1 is observed from 48 to 144 hours, implying that some denatured products are expressed. At the end of the experiment the percentage of 6-B1 stained cells is higher than observed with pPV/PV (figure IV. 13. c.). When cells transfected with pPVIII are studied (figure IV. 13. b.), two observations can be made: 1) the level of glycoprotein expression increases through cell culture but is always much lower than with pEBL1/PV or pPV/PV transfected cells; 2) whatever the day of control, transfected cells are always stained with the antibody 6-B1 whereas staining with D1 is never observed, indicating that the site III region is expressed as a denatured polypeptide.

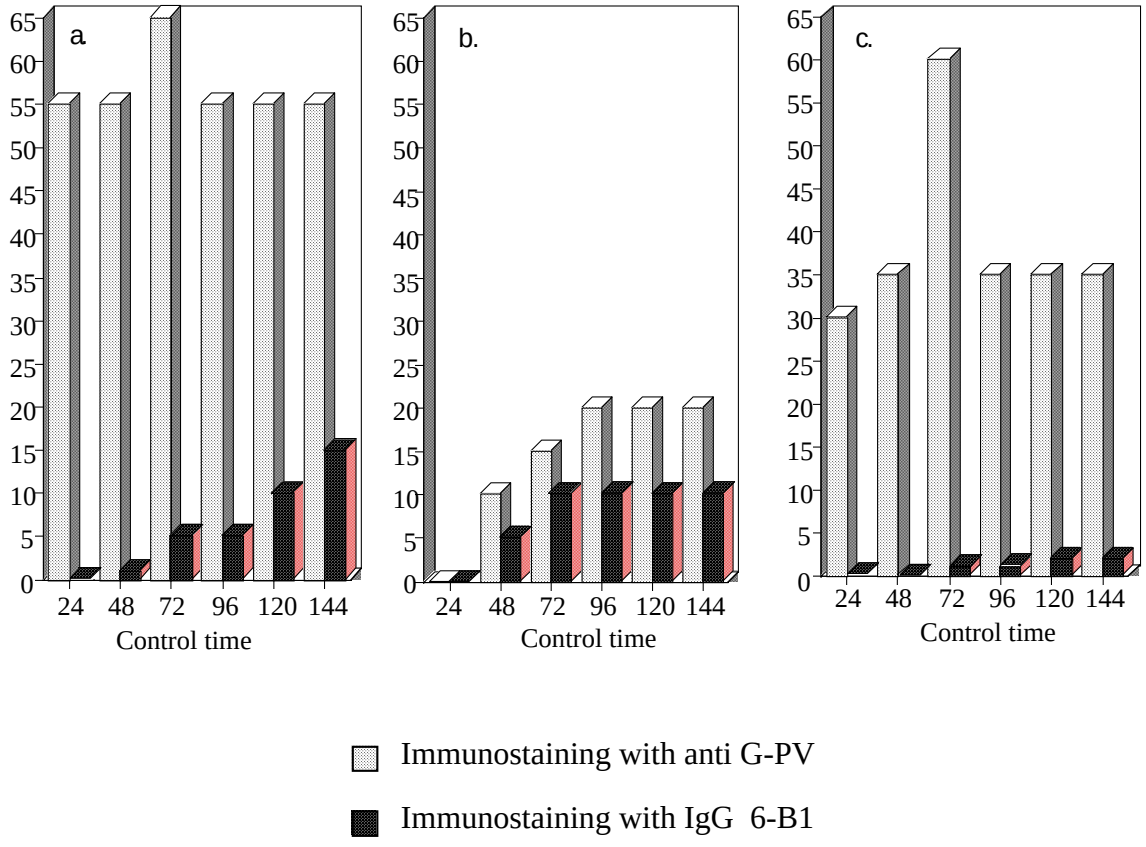


Figure IV. 13.: Recognition of cells transfected with pEBL1/PV (a.), pPVIII (b.) or pPV/PV (c.) by the polyclonal antibody anti-G PV and by the monoclonal antibody 6-B1 that is specific for denatured G. The results indicate the percentage of fluoresscent cells at different times after transfection.

When transfected cells were examined according to their morphology it was possible to distinguish two types of appearance: 1) *Foci* (F) of different size (2-20 cells) where the fluorescent cells displayed the normal irregular shape of Neuro-2a cells and the fluorescence was mainly situated on the cell membrane. This type of morphology has been observed after staining with PVE12, D1 and the polyclonal anti-G PV antibody. 2) Single cells (S) of a regular round shape with a very homogeneous fluorescence on the cell membrane and in the whole cytoplasm have been observed after staining with 6-B1 or anti-G PV (examples of typical focus and single cell fluorescence are indicated with arrows on figure IV. 12.). With homogeneous and heterogenous glycoprotein constuctions the foci morphology was predominant. Cells transfected with the truncated construction pPVIII always displayed the single cell type (table IV. 11).

In order to determine whether the expressed proteins can be released into the supernatant, the cell supernatants of pPV/PV and pPVIII transfected cells were clarified by centrifugation and tested on ELISA for the presence of glycoprotein. The supernatant of $0.3x10^6$ Neuro-2a cells transfected with pPV/PV contained 80 ng of rabies glycoprotein whereas no G has been detected in the supernatant of both, pPVIII or pCI-neo transfected cells.

In conclusion the results of *in vitro* transfection clearly indicate, that G PV and the hybrid glycoproteins Mok/PV and EBL1/PV present the epitopes II and/or III that are known to induce VNA in a similar manner as PV infected cells. The expression obtained by pVIII transfected cells mainly seems to yield an altered polypeptide.

IV. 3. 2. Immunological function

The immune response after injection of pEBL1/PV has been described elsewhere (see § IV. 3.). The truncated G, containing only the site III region (pPVIII) has been tested for its capacity to induce antibodies and Th cells.

IV. 3. 2. a. Induction of antibodies by pPVIII

As preliminary experiments indicated a very low antibody production, the plasmid injection has been accompanied by the injection of recombinant mouse IL-2 at the same site as pPVIII: IL-2 has been reported to be an important B and Th cell inducer in rabies immunity (Perrin et al., 1989) and to improve the B cell responsiveness to antigens in general (Chow et al., 1997).

pPVIII induced only a very weak antibody response, and only incombination with IL-2 (figure IV. 14.). Without coinjection of IL-2 even 100 µg of plasmid were not able to induce any measurable titres of antibodies against rabies virus. The antibody titres shown have been obtained by ELISA, VNA against PV have not been

detectable under any conditions. A reproduction of the experiment yielded the same results in two different genetic backgrounds, Balb/c and C3H mice (antibody and Th cell production; data not shown).

Consequently we assume that pPVIII was unable to induce antibodies without the artificial presence of IL-2.

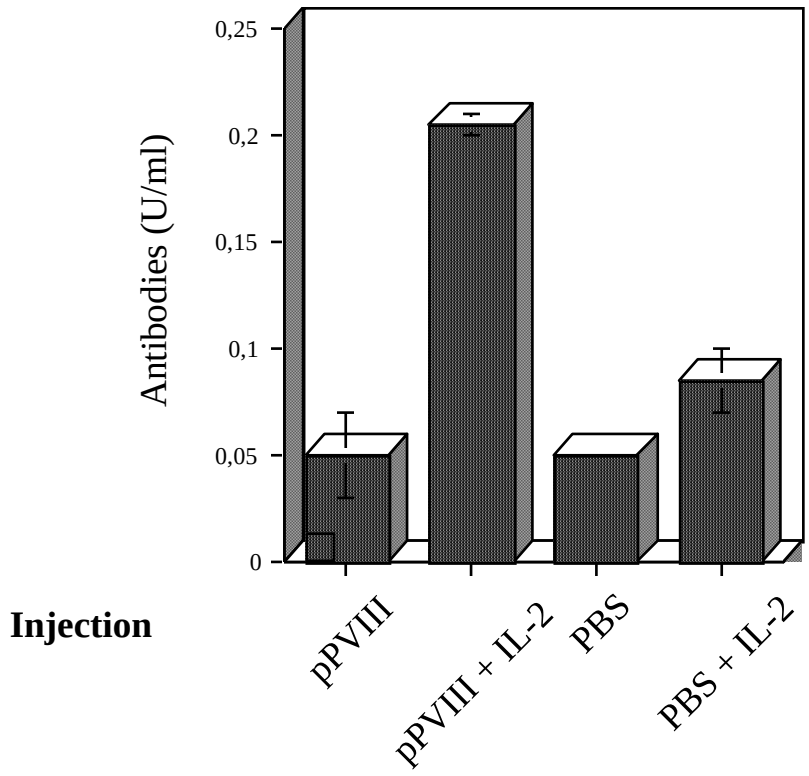


Figure IV. 14.: Induction of anti-PV antibodies (U/ml; ELISA) after injection of pPVIII. Mice were injected with 100 µg of plasmid with or without 10 U of recombinant mouse IL-2. Control mice received PBS buffer. The sera were tested on day 21.

IV. 2. 3. b. Induction of Th cells by pPVIII

Mice that have been injected with pPVIII (without coinjection of IL-2) showed a considerable Th cell response on day 21 (figure IV. 15.). After stimulation with both, IPRV and purified rabies G, the specific IL-2 production was half as high as the production induced by polyclonal stimulation with ConA.

These results clearly indicate that the presentation of pPVIII products to Th cells is as efficient as for the complete pPV/PV expression product (see § IV. 2.).

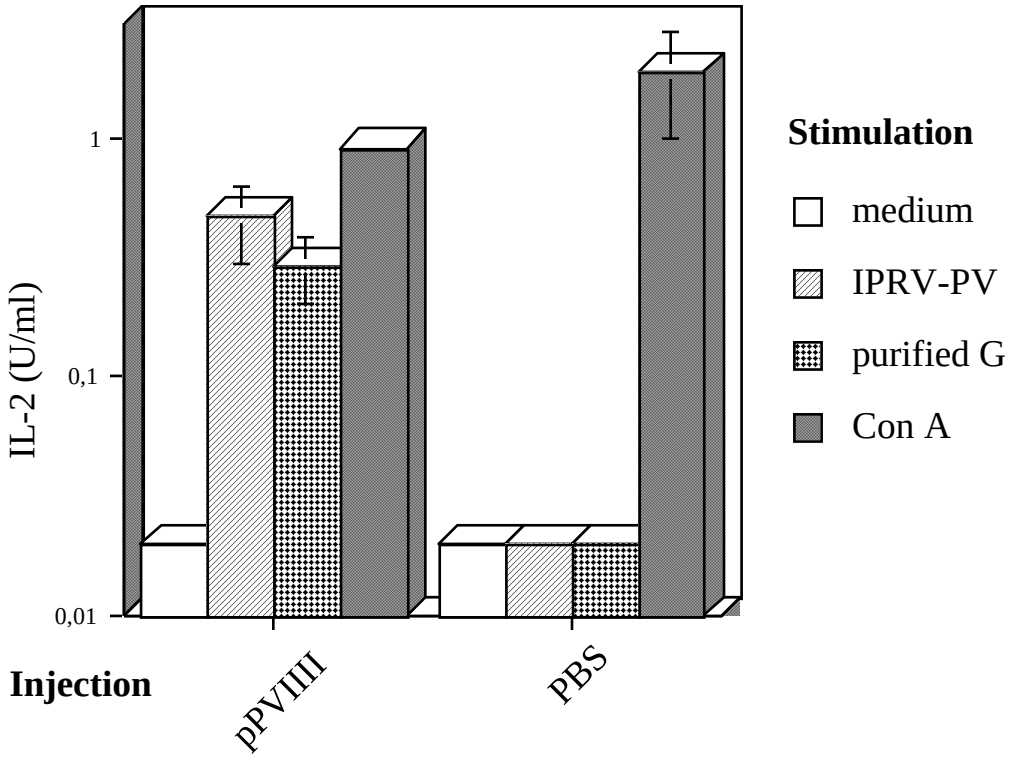


Figure IV.15.: IL-2 (U/ml) production after*in vitro* stimulation of splenocytes from mice that have been injected with 100 µg of pPVIII. Control mice received PBS. The response to stimulation with IPRV and purified G was mesured on day 21 after injection.

It can be concluded that pPVIII induces a very weak B cell responses but a high Th cell response, indicating that only Th cell epitopes are correctly presented to the immune system.

DISCUSSION

V. DISCUSSION

The aim of this study was to investigate possible approaches for an immunization against European Lyssaviruses, taking into consideration both, structural and immunological aspects.

Three lyssavirus genotypes are present in Europe: genotype 1 (classical rabies) and the genotypes 5 and 6 which represent the European bat lyssaviruses (EBL) 1 and 2. The EBLs have mainly been isolated from insectivorous bats (Amengual et al., 1997) but have as well been responsible for human cases of rabies (Lumio et al., 1986). Classical vaccines confer only partial protection against EBL1 (Lafon et al., 1988; Lafon et al., 1986; Herzog et al., 1991) and are therfore not suitable for a reliable prevention (see Introduction, § I. 2. 1. c.). Furthermore, the importance of the EBLs might increase as terrestrial rabies will probably be wiped out in Europe in consequence of massive oral vaccination campaigns for foxes. The viral equilibrium would be disturbed and the lyssaviruses might pass from aerial to terrestrial vectors as it is supposed to have been the case in the early evolution of lyssaviruses which seem to have their origin in bats (Badrane, 1997). In conclusion, future rabies immunization should preferably cover all European lyssaviruses.

The classical approach for rabies vaccination has several drawbacks: the production comprises the manipulation of viable virus and thus causes problems of security, the costs of production are very high and antigenic modification of the immunogen is nearly impossible. We have therefore chosen two new technologies and investigated their suitability for immunization against European lyssaviruses: the baculovirus expression system and DNA-based immunization. In both cases the immunization is based on the rabies virus glycoprotein (G), that has been shown to be the only rabies protein able to induce virus neutralizing antibodies (VNA; Wiktor et al., 1973) which are the crucial element of protection against rabies (Cox et al., 1977).

The baculovirus expression system (Smith et al., 1985) is a eukaryotic expression system and thus includes to a high extend the postranscriptional modifications that are characteristic for eukaryotic cells. Furthermore, this expression system has the advantage to deal with an insect cell virus that is apathogenic for vertebrates or plants. Rabies G has been successfully expressed by the baculovirus expression system and the injection of insect cells that expressed recombinant G have been able to induce protection against homologous virus challenge (Prehaud et al., 1989; Tordo et al., 1993). Nevertheless, the injection of cells would not be conceivable for vaccinal application. We therefore decided to extract G from infected cells (G-Bac). When controlled on SDS-PAGE, the size of G-Bac was slightly inferior to the size of viral G (see figure III. 1.), a phenomenon that has been reported before with rabies G expressed in insect cells and that has been explained by certain

differences in number and structure of carbohydrate side chains (Prehaud et al., 1989; Tuchiya et al., 1992). As G-Bac turned out to be unstable at 4° C, the protein solutions were lyophilized. The obtained protein was only poorly immunogenic but after association with liposomes it induced an acceptable level of VNA. A second immunogenic form of recombinant G has been obtained from insect cells: extracellular G that had been released into the supernatant, possibly by budding from the cell membrane (Gs-Bac). The immunogenicity of Gs-Bac was similar to the immunogenicity of G-Bac with liposomes. It can be assumed that in both cases the association with lipid membranes enhanced antigenicity as it has been reported with purified rabies G (Perrin et al., 1985b). Liposomes have several advantages for antigen presentation: they facilitate correct folding, they can act as adjuvant (van Rooijen N., 1990) and they improve the uptake of the antigen by macrophages (Mougin B., 1987).

As the antibody level induced by recombinant G would be insufficient to use this protein in a subunit vaccine, we have decided to associate recombinant rabies nucleoprotein (N-Bac) to the preparation in order to amplify the immunogenicity of recombinant G. The N protein plays a considerable role in anti-rabies immunity: the viral ribonucleoprotein complex (RNP) has been reported to induce protection against an intramuscular challenge (Dietzschold et al., 1989) and it is able to induce a Th cell response (Ertl, 1989b). Two ways of association of recombinant G and N have been tested: encapsulation of a mixture of G and N in liposomes or homogenization of a Gs-Bac and N mixture by ultrasonic vibration. Even though the ultrasonic treatment is a poorly controllable technique of association, it had to be chosen for Gs-Bac: this presentation of recombinant G protein completely lost its immunogenicity after lyophilization, a procedure which was necessary to afford concentrated proteins for liposome encapsulation. Both ways of association of N to G lead to an adjuvant effect. In the case of Gs-Bac this adjuvant effect clearly depended on the homogenization of the protein mixture and thus possibly on physical association of the two recombinant proteins. For the mixture of G-Bac and N-Bac the association in liposomes did not seem necessary for an adjuvant effect by N. It can be supposed, that the contaminating insect cell proteins in the G-Bac preparation mediate the association to N-Bac, whereas the interaction of Gs-Bac with other proteins is hampered by its association with membrane lipids. The quantity of N added to the G preparations seems to be important for the adjuvant effect that is observed: an optimum of immunogenicity after association of N to G has been observed with an amount of 0.1 µg N protein per injection. An adjuvant effect by N has been observed before and as N has been reported to belong to the superantigens (Lafon et al., 1992) this adjuvant effect has been explained by unspecific stimulation of Th cells by the Vβ-chain of the T cell receptor (Astoul et al., 1996). As the members of the superantigen family induce a high polyclonal Th cell response,

specific immune responses can be inhibited. It could be assumed that this is the case when high doses of recombinant N are injected, thus explaining the dose dependancy we have observed.

Another explanation for the adjuvant effect obtained with rabies nucleoprotein has been proposed by Dietzschold et coll. (Dietzschold et al., 1987b) after observation of a slight increase of rabies glycoprotein's protective activity by association with rabies RNP in liposomes. According to their hypothesis, N acts as a specific Th cell inducer and produces a cross-help phenomenon as described with influenza (Russell and Liew, 1979; see Introduction, § I. 1. 6. b.). This theory relies on B cells as antigen presenting cells for both antigens, G and N, and on N specific Th cells that stimulate the neutralizing antibody production by B cells. It would therefore explain the necessity of physical association of G and N for the observation of an adjuvant effect: only in this case the recognition by G-specific B cell receptors would induce the take up of both, G and N and the presentation of the epitopes of both proteins by the same B cell (potentially capable to produce virus neutralizing antibodies against G). The recognition of the N Th cell epitope by primed Th cells specific for the nucleoprotein would thus be accompanied by the production of antibodies against G. However, if such a cross-help effect took place in the case of rabies, N should be able to prime for an association of G and N. In a protocol of two injections (priming with N and boosting with an association of G and N) it should make a significant difference if mice were primed with N before the boost or not. In our system, we did not observe higher antibody titres when mice were primed with recombinant N before the boost with the association of recombinant G and N proteins. We have thus not been able to detect a priming effect by recombinant N. Our results are not completely surprising as the results described in literature on this subject are controversial: whereas recombinant N that has been produced by the baculovirus expression system (Fu et al., 1991) and viral RNP (Tollis et al., 1991) have been able to prime for subsequent rabies infection or vaccination, no priming has been observed with recombinant N expressed by vaccinia virus (Sumner et al., 1991) or with peptides carrying Th cell epitopes of N (Ertl et al., 1989a). It has to be noted that the interpretation of priming experiments with viral RNP is sometimes difficult, as even after purification the preparations of RNP may contain traces of G (Schneider et al., 1973; Wiktor et al., 1973) which is a potent antigen for both, B and T cells.

Our expectation was to obtain by this approach an expanded spectrum of protection due to a cross-help effect of N, since N represents a higher state of conservation among the lyssavirus genotypes than G (Bourhy et al., 1993) as well as a certain extend of cross-protection *in vivo* (Dietzschold et al., 1987b). Furthermore, the Th cell response against rabies has been shown to display cross-reactivity between the different genotypes *in vitro* (Celis et al., 1988a). But as we have not

obtained any evidence for priming by N for a homologous boost, we abandoned the idea to achieve cross-help for a heterologous boost. Furthermore, while the addition of N to a preparation of G induced an adjuvant effect concerning the VNA titres against genotype 1, only a very slight increase of VNA titres against EBL1 has been observed. Actually, the ratio of VNA titres against EBL1 and CVS decreased with the addition of N. One could suppose that the addition of N changes the presentation of G epitopes and that the epitopes that induce cross-reacting antibodies are not sufficiently exposed anymore.

It can be concluded that out of the two immunogenic presentations of recombinant G obtained by the baculovirus expression system, only the secreted G induces an acceptable level of antibodies if the protein is injected without further treatment. Recombinant G that has been extracted from the infected cells needs the association with liposomes which would be too expensive for large scale vaccine production. The association of N to recombinant G induced an adjuvant effect (possibly by its superantigen properties) concerning the genotype 1 viruses but did not produce a similar effect concerning genotype 5. Furthermore, N has not been able to prime for an association of G and N. Its addition to an immunization based on G is therefore not justified because a simple increase of the immunogenicity of recombinant G could be obtained by other means than by the addition of a second recombinant viral protein whose production would even more expensive than other adjuvants, such as biovectors (Castignolles et al., 1996). In any case, recombinant proteins do not seem to be a suitable method for an immunization covering all the different genotypes of European lyssaviruses.

The other approach that has been applied in the aim of a broadened spectrum of protection against European lyssaviruses utilized DNA-based immunization (Davis et al., 1993b). It has been shown before that DNA-based immunization with homologous glycoprotein of PV (Xiang et al., 1994; Bahloul et al., 1997) induces similar protection as classical vaccination. A heterologous construction containing the aminoterminal half of the Mokola and the carboxyterminal half of the PV glycoprotein (pMok/PV) has been able to induce protective VNA titres against all lyssavirus genotypes but Duvenhage and EBL1 (Bahloul et al., 1997), thus adding the specific protection achieved by immunization with Mokola glycoprotein to the protection obtained with the PV glycoprotein alone. These results indicate that both fragments are immunologically functional. When the calculated secondary structure of a similar hybrid of EBL1 and PV (EBL1/PV) was compared to the structure of PV/PV and Mok/PV (figure V. 1.) no remarkable differences have been noted. In all cases the site II region seems to contain a high number of β-sheets and presents a similar pattern of turns

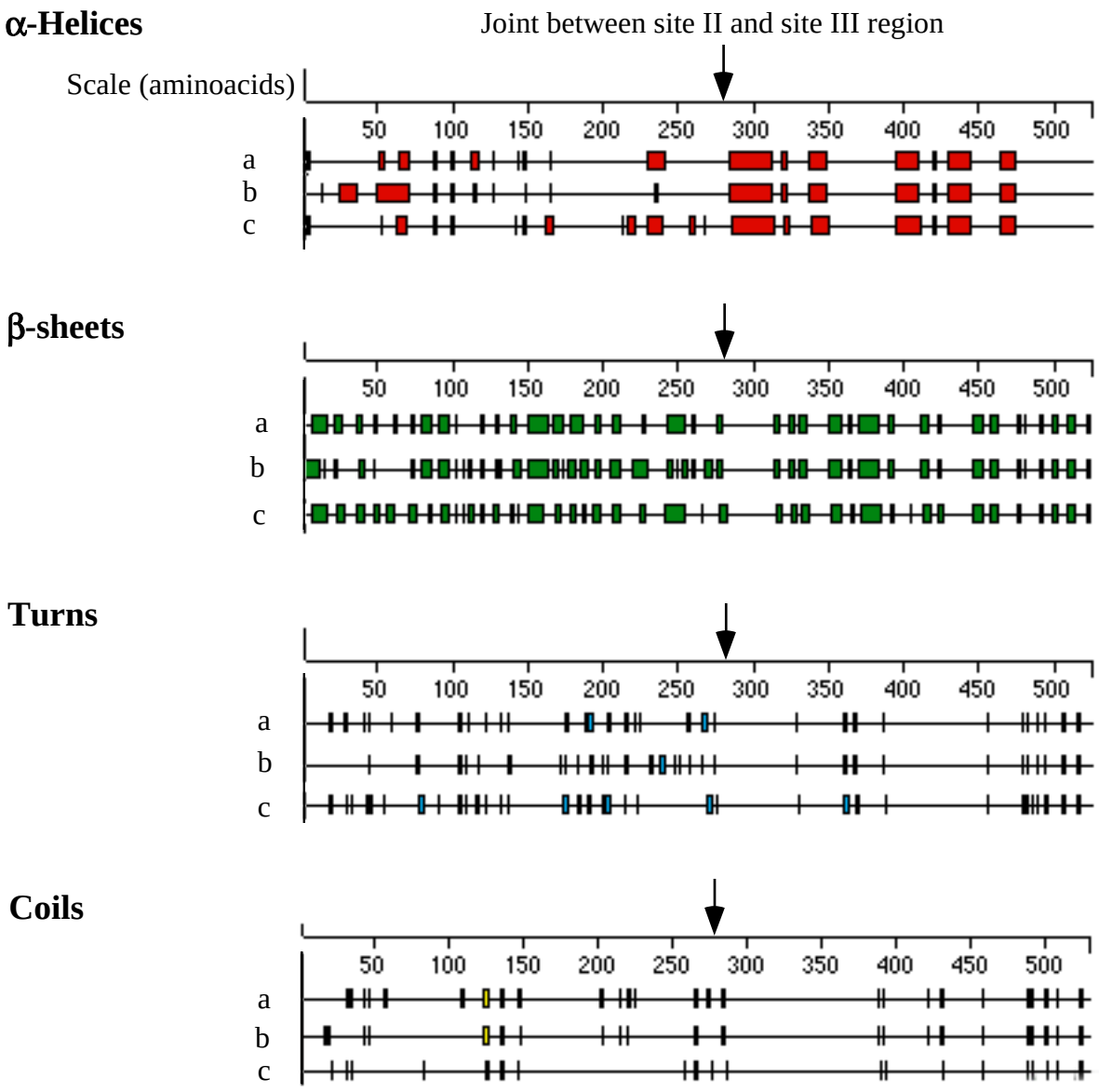


Figure V. 1.: Calculated secondary structure (Garnier-Robson) of the glyoproteins PV/PV (a), Mok/PV (b) and EBL1/PV (c).

and coils. The joint between the site II and the site III region (passage from one hybrid element to the other) is situated in a linear aminoacid sequence without B or T cell epitopes. It could therefore be expected that an EBL1/PV hybrid would express its B and T cell epitopes with the same efficiency as Mok/PV. Besides, as pEBL1/PV has been constructed with the perspective to introduce foreign epitopes in order to obtain a potential tool for transdisease immunization, two unique restriction sites have been introduced (see Materials and Methods, figure III. 5.): a site BstE II close to the aminoterminus of the protein and a site EcoR I between the site II and the site III region. The consequences of such an introduction of aminoacids within the glycoprotein will be discussed later.

DNA-based immunization with pEBL1/PV has been shown to induce a considerable Th cell response against both fragments as well as protective levels of VNA titres against both, EBL1 and PV, the titres against EBL1 being slightly superior. According to the work of Benmansour (Benmansour et al., 1991), the site II of rabies G seems to be more exposed than the site III as monoclonal antibodies against site II have been more frequently isolated than against site III. This could explain the advantage of the EBL1 site II over the PV site III concerning VNA induction. As after immunization with a genotype 1 virus a certain cross-reactivity of VNA against EBL1 is observed, one might suppose that in the case of pEBL1/PV, the PV site III region induced as well some VNA production against EBL1, thus causing the higher titre. However, this is fairly improbable as pMok/PV that contains the same PV site III region as pEBL1/PV only induces very low VNA titres against EBL1 whereas high titres against PV are observed. Furthermore, the monoclonal antibody D1 against site III of PV did not bind to EBL1 infected neuroblastoma cells (see Results, § IV. 3. 1.). Consequently, there is no antigenic homology between the site III of PV and the site III of EBL1. Similarly, the monoclonal antibody PVE12 that is directed against site II of PV did not recognize EBL1 infected cells either, reinforcing our hypothesis that in pEBL1/PV the two regions, site II and site III, act separately in VNA production. In any case, the immune response obtained with pEBL1/PV resulted in a similar protection against both, EBL1 and PV viruses, even after intracerebral challenge, demonstrating that the site III region plays a very important role in anti-rabies immunity even though the site II has always been considered to be the clearly dominating antigenic site of the rabies glycoprotein (Benmansour et al., 1991). The results obtained with DNA-based immunization with a hybrid plasmid pEBL1/PV have thus proven this approach to be superior to the classical vaccine based on inactivated Pitman Moore virus (Lafon et al., 1986) and to the protection obtained with former vaccinal approaches: 1) inactivated purified rabies virus (IPRV) PV (Lafon et al., 1988) or CVS (Herzog et al., 1991) that induced hardly more VNA against EBL1 than the Pitman Moore strain; and 2) DNA-based immunization with homogeneous glycoprotein (pPV/PV), that covered EBL1

only poorly, or with heterogenous glycoprotein (pMok/PV), that did not confer any protection against EBL1 at all (Bahloul et al., 1997). DNA-based immunization with pEBL1/PV has not yet been tested for its protective activity against EBL2. Nevertheless, even though pMok/PV did not induce protection against EBL1, it induced very high VNA titres (23 IU/ml) against EBL2. As a homologous construction pMok/Mok does not induce VNA against any member of the cross-reactive group lyssa-1 (genotype 1, 5 and 6) (Bahloul et al., 1997), the immunity against EBL2 must be due to the site III region of PV, common to pMok/PV and pEBL1/PV. It can therefore be assumed that pEBL1/PV protects as well against EBL2. Experiments are carried out at present and we expect them to confirm that immunization with pEBL1/PV induces protection against all European lyssaviruses.

The *in vitro* expression of several glycoprotein constructs carried by plasmid vectors has been investigated in comparison to the immune response induced *in vivo* in order to study the relation between structure and immunological function of the rabies glycoprotein. According to the transfected plasmid, different binding patterns of antibodies against conformational (PVE12 and D1) or against denatured epitopes (6-B1) have been observed. The morphology of the transfected cells varied between foci and single cells, depending on the expression of the transfected plasmid which is evidenced by immunostaining with characteristic antibodies. A summary of the results obtained *in vitro* and *in vivo* including Th cell response, induction of antibodies (ELISA) and of VNA (RFFIT) is shown in table V. 2. These data comprise the results obtained with two further constructs: p(C3-LCM)$_2$PVIII and pEBL1-C3-LCM-PV. For the p(C3-LCM)$_2$PVIII an insert containing a linear B cell epitope of the poliomyelitis virus, a spacer peptide containing neutral aminoacids and a linear cytotoxic T lymphocyte (CTL) epitope of the lymphocytic choriomeningitis virus (altogether 27 aminoacids) have been inserted as a dimer between the peptide signal and the site III region of PV. For pEBL1-C3-LCM-PV the same insert has been introduced as a monomer into the linear sequence between the site II and the site III region of pEBL1/PV (*).

We presume that the differences of cell morphology observed depend on the expression of the transfected plasmid. Foci seem to represent "normal" glycoprotein expression as this cell type is predominant with the expression of homogeneous glycoprotein. Furthermore, it is the only cell type observed with infected cells, the single cell form has never been detected under these conditions. Foci with up to 20 normally shaped cells cannot correspond to the actual number of glycoprotein expressing cells: at the moment of transfection, the cell layer is not confluent, transfection of a group of cells is therefore not possible. On the

* These results (E. Desmezieres) have been obtained in continuation of the research project described in this dissertation. They are citated as they provide further arguments for the discussion.

Plasmid	Localization of expression	Recognition by antibodies	Main cell morpho-logy	Th cell response	Antibodies (ELISA)	VNA
pPV/PV	Membrane	PVE12 and D1	**Foci**	+	+++	**+++**
pMok/Mok	Membrane	-	**Foci**	+	+++	**+++**
pMok/PV	Membrane	D1	**Foci**	+	+++	**+++**
pEBL1/PV	Membrane	D1	**Foci**	+	+++	**+++**
pPVIII	Membrane? Cytoplasm	6-B1	Single cells	+	+/-	**0**
pEBL1-C3-CML-PV	Membrane and cytoplasm	D1 and 6-B1	**Foci**	**+**	**++**	**++**
p(C3-CML)2-PVIII	Membrane ? Cytoplasm	D1 (weak) and 6-B1	Single cells	**+**	**+/-**	**0**

Table V. 2.: Summary of the results obtained with different glycoprotein constructs in vitro and *in vivo*.

other hand, one or two cells that are transfected could not yield a whole cluster of expressing cells as the transfected plasmid stays episomal (Wolff et al., 1992) and is thus not replicated with the cell genome during cell division. We presume that within one fluorescent focus only one or two cells express the glycoprotein that is subsequently transported to the cell membrane from where it migrates to the neighbouring cells, possibly by budding as glycoprotein has been evidenced in the supernatant of transfected cells. The sometimes filament-like appearance of the surface seen on transfected cells after antibody binding might confirm this theory. The use of neuroblastoma cells could be of importance for this phenomenon, as they carry neuronal receptors that interact specifically with certain sites of the rabies glycoprotein (Dietzschold et al., 1985). They could therefore capture the glycoprotein that has been secreted by neighbouring cells. Nevertheless, even though in the described foci G might be in a state of fusion (Perrin and Atanasiu, 1981; Gaudin and et al., 1993), we have not observed the formation of real syncytia (Morimoto et al., 1992), as the cell membranes between the different cells of a focus never disappeared.

The appearance of single cells is totally different from focus cells: they seem to be completely filled up with antigen and their round shape gives the impression of cells that have started to get detached from the cell culture support and that are possibly just about to die. As already mentioned, this form of cell morphology is mainly observed after transfection with pPVIII and it is the only cell type detected with the 6-B1 antibody that is specific for denatured glycoprotein. Following a generally shared opinion about protein synthesis and folding, only correctly folded proteins are transported to the cell membrane (Doms et al., 1993; Hurtley and Helenius, 1989). Even foreign proteins undergo this cellular control as incorrect folding is often associated with a decrease of solubility what leads to aggregation of the proteins in certain cellular compartments (Doms et al., 1987). In some cases, those incompletely or incorrectly folded proteins are associated to molecular chaperons within the endoplasmatic reticulum (ER), that retain the nascent polypeptide until its correct threedimensional structure is achieved (Ellis and Hemmingsen, 1989). It has been shown that the correct folding of the conformational sites of the rabies glycoprotein is not independent from the rest of the protein as the conformational site II could not be mimicked by peptides (Lafay et al., 1996). It can therefore be assumed that the truncated expression product of pPVIII is incorrectly folded. Furthermore, the truncated site III region lacks two out of three N-linked glycosylation sites that are present in the "full" PV glycoprotein. N-linked glycosylation helps preventing the formation of aggregates within the cell and is crucial for appropriate intracellular transport of proteins (Burger et al., 1991; Wojczyk et al., 1995). Thus the polypeptide expressed after pPVIII transfection should not be transported to the cell surface. This assumption is valid as well for any

other protein that is recognized by 6-B1. It is therefore rather surprising that immunofluorescent staining of pPVIII transfected cells or staining with 6-B1 in general can be observed without previous permeabilization of the cell membrane by fixation with acetone, indicating a protein location on the cell surface. It could be proposed that the production of incorrectly folded protein is too great to allow the normal retention by the ER. After a certain time, the cell is simply overloaded with protein and the usual control mechanisms do not work anymore, thus allowing the transport of misfolded protein to the cell surface (Y. Gaudin, personal communication). Furthermore, as the single cells seem to be dying their cell membrane might be permeabilized, allowing further secretion of the proteins and at the same time the entry of the immunofluorescent antibody, thus explaining the homogeneous fluorescence observed even with unfixed cells. The hypothesis of overexpression in the case of single cells is reinforced by the fact that single cells are a late event in the culture of transfected cells and that their frequency is rising, whereas foci are already present after 24 h and hardly increase in frequency. Besides, especially in the the case of expression of pEBL1/PV, which seems to be the most productive plasmid as it leads to the highest percentage of antigen carrying cells, the predominant morphology of foci is accompanied by a remarkable amount of single cells. It seems obvious, that especially during intense production the protein synthesis can be disturbed.

In conclusion, both types of glycoprotein expression that have been distinguished by antibody staining lead to glycoprotein transport to the cell surface, but following our hypothesis by two different mechanisms: in the case of foci glycoprotein transport occurs rather in a controlled manner, probably followed by secretion, in the case of single cells rather as a sign of cellular "anarchy" followed by protein leakage or release after lysis of dying cells. It has to be noted that the *in vitro* model with liposome-mediated transfection does not necessarily correspond to the expression of plasmid encoded antigens *in vivo* that has not yet been clearly explained.

Concerning this mechanism of DNA-based immunization it is fairly probable that after plasmid injection the immunogen is expressed by myocytes, secreted (Davis et al., 1993a) and subsequently taken up by antigen presenting cells (APC; Ertl and Xiang, 1996b). An alternative hypothesis, explaining the release of immunogen by CTL dependent cell lysis does not seem very likely as muscle cells only poorly present the MHC I complex (Karpati et al., 1988) and thus only a low CTL response and only exceptional myositis is reported after DNA-based immunization (Yokoyama et al., 1997).

If the results of *in vitro* analysis of the different plasmids are compared to the results obtained after DNA-based immunization, one is striken by the fact, that only constructs that display foci morphology and recognition by antibodies against

conformational epitopes are able to induce VNA, whereas the two constructs that always produce single cells after transfection (pPVIII and p(C3-LCM)$_2$PVIII) and are mainly recognized by 6-B1 do not induce VNA against PV and only very low levels of antibodies when measured by ELISA (table V. 2.). The modified rabies glycoprotein pEBL1-(C3-LCM)-PVIII seems to be in an intermediate position with a modified immunofluorescent staining with D1, a high frequency of single cells and induction of lower levels of VNA than after injection of pEBL1/PV. These results indicate certain changes in the folding of the site III region after introduction of a relatively small insert at distance from the site III, what is not surprising as it has been shown before with other glycoproteins that even a distant mutation can disturb the three-dimensional structure of an epitope (Parry et al., 1990). However, all constructs induce a high Th cell response that can be stimulated by inactivated purified rabies virus (IPRV), indicating that the Th cell epitope is presented in a similar manner as on the viral glycoprotein and that its functionality does not depend on the folding of the protein. This corresponds to the general observation that Th cell epitopes are mainly linear epitopes whereas B cell epitopes are generally conformational (Zinkernagel, 1996). Moreover, it has been proposed that whereas B cells only recognize paracrystalline identical antigen multimers that allow cross-linking between the epitopes by the B cell receptors, Th cells can be stimulated by monomeric antigens (Zinkernagel, 1997). Rabies glycoprotein associates to trimers as well on the virion as on the surface of infected cells. Furthermore, it has been shown in this work, that the antigen production by transfected cells of the focus type seems to induce the secretion of large, filament-like glycoprotein polymers. After transfection rabies G is therefore most probably expressed as antigen multimers which should be potent B cell antigens, as confirmed by the immune response after DNA-based immunization with full glycoprotein constructs. Concerning pPVIII and p(C3-LMV)$_2$PVIII it has to be noted that both constructs contain linear sites that should be able to induce virus neutralizing antibodies even if the site III is not correctly folded: the sites b and c and the minor site a (Prehaud et al., 1991; Benmansour et al., 1991 - see Introduction § I. 1. 7. a.). A lack of site III recognition can thus not acount for the complete absence of VNA production. But as these constructs are expressed *in vitro* in a denatured form it can be supposed that these truncated glycoproteins are not able to form oligomers or even higher polymers and that they are therefore not at all "seen" by the B cell response, whereas the Th cells are capable to recognize monomeric antigens and thus are even stimulated by these denatured polypeptides.

An additional feature of the Th cell response after DNA-based immunization can be stated: the two hybrid constructs, pEBL1/PV and pMok/PV induce a Th cell response that is easier stimulated with IPRV PV than with IPRV EBL1 or IPRV Mokola thus suggesting that the Th cell epitope situated on the site III region of the

rabies glycoprotein (see Introduction, figure I. 5. a.) is more efficient than the other G Th cell epitopes.

It can be concluded that the analysis of the *in vitro* expression of plasmid encoded rabies glycoproteins by transfected cells provides a reliable prognosis on the immunological functionality of the concerned gene product after DNA-based immunization *in vivo*. Only proteins that display typical features of correct folding and presentation *in vitro* (focus type morphology of the transfected cells and recognition by monoclonal antibodies with specificities for conformational epitopes) induce satisfactory immune responses *in vivo*, especially as far as the production of VNA is concerned. Several elements allow an estimation how far the rabies glycoprotein tolerates modification: 1) the construction of hybrid proteins, replacing one half of the glycoprotein by the corresponding aminoacids of another genotype, is well tolerated; 2) a half-length glycoprotein containing the site III region is only expressed in a denatured form, but maintains its properties as a Th cell inductor; 3) the introduction of linear epitopes in the middle of the rabies glycoprotein leads to an intermediate situation: the expression *in vitro* is modified and the VNA production *in vivo* decreases but is still at protective levels. These results indicate that the immunological function of the rabies glycoprotein to induce VNA is closely linked to its structure and that G only tolerates limited modifications. Introduction of a peptide of 27 aminoacids, even at a region of the glycoprotein with a linear secondary structure induces a certain perturbation of structure and function. In the contrary, the Th cell epitopes on the site III region of G tolerate all described modifications, indicating an important role of the site III region concerning the immune response against homologous and heterelogous glycoproteins.

Perspectives

Among various perspectives, we propose two fields for the continuation of this work: 1) vaccinology with the development of multivalent vaccines, and 2) fundamental immunology with the study of the relation between glycoprotein structure and its immunological function.

Concerning the field of vaccinology, the plasmid pEBL1/PV would so far not be a candidate for vaccination of humans as DNA-based immunization still carries too many risks for its application in human medicine. However, it could be tested for its performances as a veterinary vaccine, for example in dogs. For wildlife, the use of DNA-based vaccination is not thinkable as this technique requires intramuscular injection of the plasmid. But mucosal delivery of DNA has already been attempted and at length oral immunization might be possible using acidoresistant microspheres

(Gao et al., 1997). In terms of a multivalent vaccine conferring immunity against different infectious diseases by one single plasmid construct, two approaches can be imagined, using either pEBL1/PV or pPVIII as carrier of the foreign epitopes. The results we have obtained so far with pEBL1/PV as carrier have been encouraging as the insertion of a small peptide in the middle of the full hybrid glycoprotein only slightly disturbed both, its structure and immunogenicity. At the moment it is investigated, which length of insertion is tolerated before the glycoprotein looses its capacity to induce virus neutralizing antibodies against rabies. It should be worth testing, whether an insertion in the other unique restriction site which is situated on the aminoterminal extremity of the hybrid protein would be more suitable for the introduction of additional aminoacid sequences. Alternatively, pPVIII might as well be used as a carrier ("träger") of larger foreign antigenic sites, which could be inserted in the middle of the conformational hybrid protein EBL1/PV. pPVIII would be interesting for this purpose, not for its weak immunogenicity concerning rabies but for its properties as a Th cell inductor able to provide help for the foreign polypeptide.

In the field of fundamental immunology the *in vitro* expression of different native and modified rabies glycoproteins should be analysed more in detail in order to compare their protein structure and to study for example the influence of glycosylation and oligomerization on the immunological function of rabies glycoprotein

CONCLUSION
AND
SUMMARY

VI. CONCLUSION

Classical vaccines protect only partially against the European bat lyssaviruses which have been responsible for sporadic cases of fatal rabies in humans and whose importance as a reservoir for terrestrial rabies cannot be ruled out.

Among two approaches, recombinant rabies proteins and DNA-based immunization, that have been tested with the intention to obtain a rabies immunization with a broadened spectrum against the European bat lyssaviruses, only DNA-based immunization has yielded satisfactory results. In fact, the immune response obtained with recombinant rabies glycoprotein produced by the baculovirus expression system was only moderate even after addition of recombinant nucleoprotein. In the contrary, a plasmid coding for a hybrid glycoprotein containing the aminoterminal half of the EBL1 glycoprotein with the antigenic site II and the carboxyterminal half of the PV glycoprotein with the antigenic site III was able to induce protection against both parental viruses, and should, according to previous results, protect as well against the European bat lyssavirus 2. This plasmid does therefore represent a concept if not a prototype of a vaccine against the European lyssaviruses which could be used for example in dogs.

A truncated glycoprotein, containing only the site III region of the Pasteur virus, is a potent Th cell inductor and thus a potential carrier of foreign epitopes. On the other hand, it does not induce a significant B cell response, whereas the addition of the site II region of European bat lyssavirus 1 does not only reestablish the conformational site III but even enables it to induce independently a similarly high level of protection against the Pasteur virus as the EBL1 site II against EBL1. These results establish the important role of the site III region in rabies immunity whereas, site II has always been considered to be the clearly dominant antigenic site of the rabies glycoprotein (Benmansour et al., 1991).

The comparison between the *in vitro* expression of glycoprotein constructs and their stimulation of the immune response *in vivo*, has shown that this modell of double analysis can be used to study the relation of rabies glycoprotein structure and its immunological function.

Summary

Towards a vaccine against the European Lyssaviruses - a structural and immunological approach

Rabies and rabies-related viruses can infect all warm-blooded animals where they cause fatal encephalomyelitis. In Europe, apart from classical rabies mainly present in foxes, the European bat lyssaviruses (EBL) 1 and 2 have been isolated and, sporadically, they have been responsible for fatal cases in humans. The commercial vaccines which protect against classical rabies cover only partially these rabies-related viruses, whose potential importance as a reservoir for terrestrial rabies cannot be ruled out. In this work, two approaches of immunization against rabies have been investigated for their capacity to broaden the protection against the European bat lyssaviruses: immunization with recombinant rabies proteins and immunization with "naked" DNA (DNA-based immunization).

Recombinant rabies glycoprotein produced by the baculovirus expression system only induces moderate levels of neutralizing antibodies. The addition of recombinant nucleoprotein has an adjuvant effect on the production of virus neutralizing antibodies concerning classical rabies. In opposition to several publications, neither a cross-help effect or priming nor an adjuvant effect concerning the EBLs is observed. The approach of recombinant proteins has therefore turned out to be dissatisfactory for our purposes.

On the contrary, DNA-based immunization with a plasmid coding for a hybrid glycoprotein (pEBL1/PV) induces a potent immune response. This hybrid construction contains: 1) the aminoterminal half of the EBL 1 glycoprotein including the antigenic site II and 2) the carboxyterminal half of the Pasteur virus (PV) glycoprotein which comprises the antigenic site III. pEBL1/PV is able to induce T helper cells and virus neutralizing antibodies and to raise a protection against both parental viruses. According to previous results pEBL1/PV should protect as well against the EBL 2; it can therefore be proposed as a possible vaccine against all European lyssaviruses.

A plasmid coding for a truncated glycoprotein (pPVIII) containing only the carboxyterminal half with the site III is able to induce a T helper cell response at a similar level as the "full" glycoprotein. The B cell epitope is only functional in a full glycoprotein (pEBL1/PV) where it induces a similarly high level of protection against PV as the EBL1 site II against EBL1 thus demonstrating the important role of the site III region in rabies immunity.

A comparison between the *in vitro* expression of different glycoprotein constructions and their immunogenicity *in vivo* shows that the induction of virus neutralizing antibodies depends on the correct folding of the glycoprotein, whereas even incorrectly folded glycoproteins are able to induce a potent Th cell response. Thus this model of double analysis can be used to study the relation between the quality of the *in vitro* expression and the immunological function *in vivo* of rabies glycoprotein.

Zusammenfassung

Impfung gegen die Europäischen Lyssaviren - ein struktureller und immunologischer Ansatz

Tollwut und die Tollwut-verwandten Viren sind potentielle Erreger einer tödlichen Enzephalomyelitis bei allen Warmblütern. In Europa wurden außer der hauptsächlich in Füchsen vorkommenden klassischen Tollwut die europäischen Fledermauslyssaviren (European bat lyssaviruses, EBL) 1 und 2 isoliert, die aber auch schon für einige Todesfälle beim Menschen verantwortlich waren. Die auf dem Markt erhältlichen Impfstoffe schützen zwar gut gegen klassische Tollwut, decken jedoch diese Tollwut-verwandten Viren, deren Rolle als Reservoir für die Fuchstollwut nicht ausgeschlossen werden kann, nur teilweise ab. In der vorliegenden Arbeit wurden zwei Ansätze von Tollwutimmunisierung auf ihre Fähigkeit hin untersucht, den Impfschutz gegenüber den EBLs zu erweitern: Immunisierung mit rekombinanten Tollwutproteinen und Immunisierung mit "nackter" DNA (DNA-Immunisierung).

Rekombinantes Tollwutglykoprotein aus dem Baculovirus-Expressionssystem induziert nur niedrige Antikörpertiter. Das Hinzufügen von rekombinantem Nukleoprotein bewirkt einen Adjuvans-Effekt bezüglich der Produktion von neutralisierenden Antikörpern gegen klassische Tollwut. In Widerspruch zu einigen Veröffentlichungen kann in unserem System weder ein Cross-help oder Priming-Effekt noch ein Adjuvans-Effekt gegenüber den EBLs nachgewiesen werden. Der Ansatz der rekombinanten Proteine mußte daher als unzureichend für unsere Zwecke angesehen werden.

Im Gegensatz dazu kann DNA-Immunisierung mit einem Plasmid, das für ein Hybridglykoprotein kodiert (pEBL1/PV), eine ausgeprägte Immunantwort induzieren. Diese Hybridkonstruktion enthält: 1) die aminoterminale Hälfte des EBL1-Glykoproteins mit dem Epitop II und 2) die carboxyterminale Hälfte des Pasteur Virus (PV) Glykoproteins mit dem Epitop III. pEBL1/PV ist in der Lage, T-Helfer-Zellen und neutralisierende Antikörper zu induzieren und einen Immunschutz gegen beide Viren aufzubauen. Da aufgrund von vorhergehenden Ergebnissen davon ausgegangen werden kann, daß pEBL1/PV auch gegen EBL2 schützt, kann dieses Plasmid als ein möglicher Impfstoff gegen sämtliche europäische Lyssaviren angesehen werden.

Ein für ein verkürztes Glykoprotein (pPVIII) kodierendes Plasmid, das nur die carboxyterminale Hälfte des Proteins mit dem Epitop III enthält, ist in der Lage, eine T-Helferzell-Antwort in einer ähnlichen Stärke wie das vollständige Glykoprotein zu induzieren. Das B-Zell-Epitop ist nur in einem vollständigen Glykoprotein (pEBL1/PV) funktionsfähig; in diesem Fall ist es in der Lage, einen ähnlich hohen Schutz gegen PV zu induzieren wie das EBL1-Epitop II gegen EBL1, was die bedeutende Rolle der Epitop III-Region in der Immunität gegen Tollwut demonstriert.

Ein Vergleich zwischen der *in vitro* Expression verschiedener Glykoproteine mit ihrer Immunogenität *in vivo* zeigt, daß die Induktion neutralisierender Antikörper eine korrekte Faltung des Glykoproteins erfordert, wohingegen selbst Glykoproteine mit einer stark abweichenden Faltung eine hohe T-Zell-Antwort induzieren können. Unser Modell einer doppelten Analyse ist somit dazu geeignet, die Beziehung zwischen der Qualtität der *in vitro* Expression und der immunologischen Funktion *in vivo* zu untersuchen.

Résumé

Vers un vaccin contre les Lyssavirus européens - une approche structurale et immunologique

La rage et les virus apparentés sont capables d'infecter tous les animaux à sang chaud où ils sont à l'origine d'une encéphalomyélite mortelle. En Europe on a pu isoler le virus de la rage classique surtout porté par les renards ainsi que les lyssavirus des chauves-souris européennes (European bat lyssavirus, EBL) 1 et 2 qui ont eux aussi été responsables de quelques cas humains. Les vaccins commerciaux protègent contre la rage classique mais ne couvrent que partiellement les virus EBL dont l'importance en tant que réservoir de la rage terrestre ne peut pas être négligée. Dans ce travail, deux approches d'immunisation anti-rabique ont été étudiées pour leur capacité à élargir le spectre de protection contre les virus EBL: immunisation avec des protéines rabiques recombinantes et l'immunisation avec l'ADN "nu" (immunisation ADN).

Les protéines rabiques recombinantes produites via le système d'expression dérivé des baculovirus n'induit qu'un niveau modéré d'anticorps neutralisants. L'addition de la nucléoprotéine recombinante induit un effet adjuvant sur la production d'anticorps neutralisants dirigés contre le virus de la rage. Contrairement à ce qui a été écrit dans quelques publications, on ne peut observer ni d'effet de "cross-help" ou de "priming" ni d'effet adjuvant pour les virus EBL. La téchnique des protéines recombinantes s'est donc avérée insuffisante dans notre cas.

Au contraire, l'immunisation ADN avec un plasmide qui code pour une glycoprotéine hybride (pEBL1/PV) induit une réponse immunitaire importante. Cette construction hybride contient: 1) la moitié aminoterminale de la glycoprotéine EBL1 comprenant le site antigénique II et 2) la moitié carboxyterminale de la glycoprotéine du virus Pasteur (PV) y compris le site antigénique III. pEBL1/PV est capable d'induire des cellule T "helper" et des anticorps neutralisants et d'aboutir à une protection contre chacun des deux virus parentaux. D’après des resultats antérieurs il est probable que pEBL1/PV puisse protéger aussi contre le virus EBL2. Ce plasmide peut donc être proposé comme vaccin contre tous les lyssavirus Européens.

Un plasmide codant pour une glycoprotéine tronquée (pPVIII) qui ne contient que la moitié carboxyterminale avec le site III s'est avérée induire une réponse T "helper" d'un niveau similaire à la glycoprotéine complète. L'épitope B n'est fonctionnel que dans une protéine complète (pEBL1/PV) où il est capable d'induire une protection contre PV du même niveau que la protection contre EBL1 induite par le site II EBL1. Ceci met en évidence le rôle important de la région site III dans l'immunité rabique.

Une comparaison entre l'expression *in vitro* de différentes constructions de glycoprotéine et leur immunogénicité *in vivo* permet de démontrer que l'induction d'anticorps neutralisants dépend du "folding" correct de la glycoprotéine tandis qu’une glycoprotéine même mal foldée peut induire une réponse T "helper" importante. Ce double niveau d'analyse peut donc être utilisé pour étudier la relation entre la qualité de l'expression *in vitro* et la fonction immunologique de la glycoprotéine rabique.

REFERENCES

REFERENCES

Acha-Orbea, H. (1992). Retroviral superantigens. *Chem. Immunol.* **55,** 65-86.

Acha-Orbea, H., Held, W., Waanders, G. A., Shakhov, A. N., Scarpellino, L., Lees, R. K., and MacDonald, H. R. (1993). Exogenous and endogenous mouse mammary tumour virus superantigens. *Immunol. Rev.* **131,** 5-25.

Amengual, B., Whitby, J. E., King, A., Cobo, J. C., and Bourhy, H. (1997). Evolution of European bat lyssaviruses. *J. Gen. Virol.* **78,** 2319-2328.

Anderson, R., Gao, X., Papakonstantinopoulou, A., Roberts, M., and Dougan, G. (1996). Immune response in mice following immunization with DNA encoding fragment C of tetanus toxin. *Infect. Immun.* **64,** 3168-3173.

Andral, B., and Blacou, J. (1981). Study of the mechanisms of early death occuring after vaccination in mice inoculated with street rabies virus. *Ann. Virol. (Inst. Pasteur)* **132 E,** 503-517.

Anilionis, A., Wunner, W. H., and Curtis, P. J. (1981). Structure of the glycoprotein gene in rabies virus. *Nature* **294,** 275-278.

Artois, M., Guittré, I., Leblois, H., Brochier, B., and Barrat, J. (1992). Potential pathogenicity for rodents of vaccines intended for oral vaccination against rabies: a comparison. *Vaccine* **10,** 524-528

Astoul, E., Lafage, M., and Lafon, M. (1996). Rabies superantigen as a Vb T-dependent adjuvant. *J. exp. Med.* **183,** 1623-1631.

Atanasiu, P., Tsiang, H., Perrin, P., Favre, S., and Sisman, J. (1974). Pouvoir immunisant d'un antigène soluble (glycoprotéine) du virus rabique purifié, traité par le triton X 100. *C. R. Acad. Sc. Paris.* **279D,** 875-878.

Atanasiu, P., Arita, M., Perrin, P., Manganas, O., and Sureau, P. (1981). Propriétés physicochimiques et antigéniques du virus rabique. *C. R. Acad. Sc. Paris.* **293.III,** 43-49.

Badrane, H., Bahloul, C., Kissi, B., Bourhy, H., and Tordo, N. (1997). Genetic diversity of lyssaviruses: implications in vaccinology, pathology and phylogeny. *submitted*

Baer, G., Shaddock, J., Levy, H., and Johnston, P. (1988). Interferon in post-exposure prophylaxis. *In "Progress in rabies control"* (O. Traenhart, H. Koprowski, K. Bögel and Pierre Sureau Eds), 245-250. Wells Medical,

Baer, G. M., Bellini, W. J., and Fishbein, D. B. (1990a). Rhabdoviruses. *In "Virology* " (B. N. Fields and D. M. Knipe Eds), 883, Raven Press, Ltd., New York.

Baer, G. M., Shaddock, J. H., Quirion, R., Dam, T. V., and Lentz, T. L. (1990b). Rabies susceptibility and acetylcholine receptor. *Lancet* **335,** 664-665.

Bahloul, C., Jacob, Y., Tordo, N., and Perrin, P. (1997). DNA-based immunization for exploring the enlargement of immunological cross-reactivity against the lyssaviruses. *Vaccine (accepted)*

Bailey, M. J., McLeod, D. A., Kang, C.-Y., and Bishop, D. H. L. (1989). Glycosylation is not required for the fusion activity of the G protein of vesicular stomatitis virus in insect cells. *Virology* **169,** 323-331.

Ban, E. (1997). New virus leaves questions hanging. *Nature Medicine* **3,** 5.

Bangham, A. D., Standish, M. M. and Watkins, J. C. (1965). Diffusion of univalent ions across the lamellae of swollen phospholipids. *J. Mol. Bio.* **13,** 238-258.

Baumann, O., Walz, B., Somlyo, A. V., and Somlyo, A. P. (1991). Electron probe microanalysis of calcium release and magnesium uptake by endoplasmic reticulum in bee photoreceptors. *Proc. Natl. Acad. Sci. USA* **88,** 741-744.

Benmansour, A., Leblois, H., Coulon, P., Tuffereau, C., Gaudin, Y., Flamand, A., and Lafay, F. (1991). Antigenicity of rabies virus glycoprotein. *J. Virol.* **65,** 4198-4203.

Bergmann, J. E., Tokuyasu, K. T., and Singer, S. J. (1981). Passage of an integral membrane protein, the vesicular stomatitis virus glycoprotein, through the Golgi apparatus en route to the plasma membrane. *Proc. Natl. Acad. Sci. USA* **78,** 1746-1750.

Bierer, B. E., Sleckman, B. P., Ratnofsky, S. E., and Burakoff, S. J. (1989). The biologic roles of CD2, CD4, and CD8 in T-cell activation. *Ann. Rev. Immunol.* **7,** 579-599.

Biron, C. A. (1994). Cytokines in the generation of immune response to, and resolution of, virus infection.*Current Opinion in Immunology* **6,** 530-538.

Bourhy, H., Kissi, B., Lafon, M., Sacramento, D., and Tordo, N. (1992). Antigenic and molecular characterization of bat rabies virus in Europe. *J. Clin. Microbiol.* **30,** 2419-2426.

Bourhy, H., Kissi, B., and Tordo, N. (1993). Molecular diversity of the lyssavirus genus. *Virology* **194,** 70-81.

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

Bunschoten, H., Gor, M., Claassen, I. J. T. M., Uytedehaag, F. G. C. M., Dietzschold, B., Wunner, W. H., and Osterhaus, A. D. M. E. (1989). Characterization of a new virus-neutralizing epitope that denotes a sequential determinant on the rabies virus glycoprotein. *J. Gen. Virol.* **70,** 291-298.

Burger, S. R., Remaley, A. T., Danley, J. M., Moore, J., Muschel, R. J., Wunner, W. H., and Spitalnik, S. L. (1991). Stable expression of rabies virus glycoprotein in chinese hamster ovary cells. *J. Gen. Virol.* **72,** 359-367.

Cadoz, M., Strady, A., Meignier, B., Taylor, J., Tartaglia, J., Paoletti, E., and Plotkin, S. (1992). Immunisation with canarypox virus expressing rabies glycoprotein. *The Lancet* **339,** 1429-1432.

Castignolles, N., Morgeaux, S., Gontier-Jallet, C., Samain, D., Betbeder, D., and Perrin, P. (1996). A new family of carriers (Biovectors) enhances the immunogenicity of rabies antigens. *Vaccine* **14,** 1353-1360.

Celis, E., Wiktor, T., Dietzschold, B., and Koprowski, H. (1985). Amplification of rabies virus-induced stimulation of human T-cell lines and clones by antigen-specific antibody. *J. Virol.* **56,** 426-433.

Celis, E., Miller, R., Wiktor, T., Dietzschold, B., and Koprowski, H. (1986). Isolation and characterization of human T cell lines and clones reactive to rabies virus: antigen specificity and production of interferon gamma. *J. Immunol.* **136,** 692-697.

Celis, E., Kari, R., Dietzschold, B., Wunner, W., and Koprowski, H. (1988a). Genetic restriction and fine specificity of human T cell clones reactive with rabies virus. *J.Immunol.* **141,** 2721.

Celis, E., Ou, D., Dietzschold, B., and Koprowski, H. (1988b). Recognition of rabies and rabies-related viruses by T cell derived from human vaccine recipients. *J. Virol.* **62,** 3128-3134.

Celis, E., Ou, D., Dietzschold, B., Otvos, L., and Koprowski, H. (1989). Rabies virus-specific T cell hybridomas: identification of class II MHC-Restricted T-cell epitopes using synthetic peptides. *Hybridoma* **8,** 263-275.

Celis, E., Rupprecht, C. E., and Plotkin, S. A. (1990). New and improved vaccines against rabies. *In "New Generation Vaccines"* (G. C. Woodrow and M. M. Levine Eds), 419-438. Dekker, M., New York, Basel.

Charlton, K. M. (1994). The pathogenesis of rabies and other lyssaviral infections: recent studies. *In "Lyssaviruses"* (C. E. Ruprecht, B. Dietzschold and H. Koprowski Eds),95-120. Springer-Verlag, Berlin, Heidelberg, New York

Chenik, M., Chebli, K., Gaudin, Y., and Blondel, D. (1994). *In vivo* interaction of rabies virus phosphoprotein (P) and nucleoprotein (N): existence of two N-binding sites on P protein. *J. Gen. Virol.* **75,** 2889-2896.

Cho, S., Narahara, H., Mifune, K., and Kawai, A. (1987). Murine T cell clones directed to rabies virus: isolation and some of their properties. *J. Gen. Virol.* **68,** 1115-1123.

Chow, Y.-H., Huang, W.-L., Chi, W.-K., Chu, Y.-D., and Tao, M.-H. (1997). Improvement of hepatitis B virus DNA vaccines by plasmids coexpressing hepatitis B surface antigen and interleukin-2. *J. Virol.* **71,** 169-178.

Clark, J. M. (1988). *Nucl. Acids Res.* **16,** 9677-9686.

Cole, B. C., and Atkins, C. L. (1991). The Mycoplasma athritidis T cell mitogen, MAML a model superantigen. *Immunol. Today* **12,** 271.

Compans, R., and Choppin, P. (1967). The length of the helical nucleocapsid of Newcastle disease virus. *Virology* **33,** 344-346.

Costa, G. L., and Weiner, M. P. Polishing with T4 or Pfu polymerase increases the efficiency of cloning of PCR fragments. *Nucl. Acids Res*. **22**, 2423

Couterlier, J.-P., van der Logt, J. T. M., Heessen, F. W. A., Vink, A., and Snick, J. (1988). Virally induced modulation of murine IgG antibody subclasses. *J. Exp. Med.* **168,** 2373-2378.

Cox, J., Dietschold, B., and Schneider, L. (1977). Rabies virus glycoprotein. Biological and serological characterization. *Infect. Immunity.* **16,** 754-759.

Cox, J., Dietzschold, B., Weiland, F., and Scheider, L. (1980). Preparation and characterization of rabies virus hemagglutinin. *Infect. and Immun.* **30,** 572-577.

Davis, D. R., and Metzger, H. (1983). Structural basis of antibody function. *Annu. Rev. Immunol.* **1,** 87-147.

Davis, H. L., Michel, M.-L., and Whalen, R. G. (1993a). DNA-based immunization induces continous secretion of hepatitis B surface antigen and high levels of circulating antibody. *Human. Molec. Genet.* **2,** 1847-1851.

Davis, H. L., Whalen, R. G., and Demeneix, B. (1993b). Direct gene transfer into skeletal muscle *in vivo*: factors affecting efficiency of transfer and stability of expression. *Hum. Gene Ther.* **4,** 151-159.

Davis, H. L., Michel, M. L., Mancini, M., Schleef, M., and Whalen, R. G. (1994). Direct gene transfer in skeletal muscle: plasmid DNA-based immunization against the hepatitis B virus surface antigen. *Vaccine* **12,** 1503-1509.

Delagneau, J. F., Perrin, P., and Atanasiu, P. (1981). Structure of the rabies virus: spatial relationships of the proteins G, M1, M2 and N. *Ann. Virol. (Inst. Pasteur).* **132E,** 473-493.

Dellabona P., Peccoud, J., Kappler J., Marrack P., Benoist C., and Mathis D. (1990). Superantigens interact with MHC class II molecules outside the binding groove. *Cell* **62,** 1115-1121.

Devauchelle, G., and Cerutti, M. (1993). Les Baculovirus d'insectes: vecteur d'expression de gênes étrangers. *Regard sur la Biochimie* **1**, 57-65.

Dietzschold, B., Wiktor, T. J., Macfarlan, R., and Varrichio, A. (1982). Antigenic structure of rabies virus glycoprotein: ordering and immunological characterization of large CVBr-cleavage fragments. *J. Virol.* **44,** 595-602.

Dietzschold, B., Wiktor, T., Wunner, W., and Varrichio, A. (1983a). Chemical and immunological analysis of the rabies soluble glycoprotein. *Virology* **124,** 330-337.

Dietzschold, B., Wunner, W., Wiktor, T., Lopes, D., Lafon, M., Smith, C., and Koprowski, H. (1983b). Characterization of an antigenic determinant of the glycoprotein that correlates with pathogenicity of rabies virus. *Proc. Natl. Acad. Sci. USA.* **80,** 70-74.

Dietzschold, B., Wiktor, T., Trojanowski, J., MacFarlan, R., Wunner, W., Torres-anjel, M., and Koprowski, H. (1985). Differences in cell-to-cell spread of pathogenic and apathogenic rabies virus *in vivo* and *in vitro*. *J. Virol.* **56,** 12-18.

Dietzschold, B., Lafon, M., Wang, H., Otvos, L., Celis, E., Wunner, W., and Koprowski, H. (1987a). Localization and immunological characterization of antigenic domains of the rabies virus internal N and NS proteins. *Virus Research* **8,** 103-125.

Dietzschold, B., Wang, H., Rupprecht, C. E., Celis, E., Tollis, M., Ertl, H., Heber-Katz, E., and Koprowski, H. (1987b). Induction of protective immunity agaist rabies by immunisation with rabies virus ribonucleoprotein. *Proc. Natl. Acad. Sci. USA* **84,** 9165-9169.

Dietzschold, B., Rupprecht, C., Tollis, M., Lafon, M., Mattei, J., Tadeusz, J. W., and Koprowski, H. (1988). Antigenic diversity of the glycoprotein and nucleocapsid protein of rabies and rabies related virus. *Rev. infect. Dis. (Suppl. 4)* **10,** 785-798.

Dietzschold, B., Gore, M., Ertl, H., Celis, E., Otvos, L. J., and Koprowski, H. (1989). Analysis of protective immune mechanisms induced by rabies nucleoprotein. In „Genetics and pathogenicity of negative strand viruses“ (D. Kolakowski and B. Mahy Eds.), 295-305. Elesevier Science Publishers BV.

Dietzschold, B., Gore, M., Casali, P., Ueki, Y., Rupprecht, C., Notkins, A., and Koprowski, H. (1990a). Biological characterization of human monoclonal antibodies to rabies virus. *J. Virol.* **64,** 3087-3090.

Dietzschold, B., Gore, M., Marchadier, D., Niu, H.-S., Bunschoten, H. M., Otvos, L. J., Wunner, W. H., Ertl, H. C. J., Osterhaus, A. D. M. E., and Koprowski, H. (1990b). Structural and immunological characterization of a linear virus-neutralizing epitope of the rabies virus glycoprotein and its possible use in a synthetic vaccine. *J. Virol.* **64,** 3804-3809.

Doms, R. W., Helenius, A., and Balch, W. (1987). Role for ATP in the assembly and transport of VSV G protein trimers. *J. Cell. Biol.* **105,** 1957-1969.

Doms, R. W., Lamb, R. A., Rose, J. K., and Helenius, A. (1993). Folding and assembly of viral membranes proteins. *Virology* **193,** 545-562.

Durrer, P., Gaudin, Y., Ruigrok, R. W. H., Graf, R., and Brunner, J. (1995). Photolabeling identifies a putative fusion domain in the envelope glycoprotein of rabies and vesicular stomatitis virus. *J. Biol. Chem.* **270,** 17575-17581.

Ellis, R. J., and Hemmingsen, S. M. (1989). Molecular chaperons: proteins essential for the biogenesis of some macromolecular structures. *Trends Biochem. Sci.* **14,** 339-342.

Ertl, H. (1989a). Dominant T-helper cell epitopes of the rabies virus nucleoprotein. *In "Rabies* " (O. Thraenhart Ed.), 238-242. Wells Medical, Kent

Ertl, H., Dietzschold, B., Gore, M., Otvos, L., Larson, J., Wunner, W., and Koprowski, H. (1989b). Induction of rabies virus-specific T-helper cells by synthetic peptides that carry dominant T-helper cell epitopes of the viral ribonucleoprotein. *J. Virol.* **63,** 2885-2892.

Ertl, H., Dietzschold, B., and Otvos, L. (1991). T helper epitope of rabies virus nucleoprotein defined by tri and tetrapeptides. *Eur. J. Immunol.* **21,** 1-10.

Ertl, H., and Xiang, Z. (1996a). Novel vaccine approaches. *J. Immunol.* **156,** 3579-3582.

Ertl, H. C. J., and Xiang, Z. Q. (1996b). Genetic Immunization. *Viral Immunology* **9,** 1-9.

Fekadu, M., Shaddock, J., Sanderlin, D., and Smith, J. (1988). Efficacy of rabies vaccines against Duvenhage virus isolated from European house bats (Eptesicus serotinus), classic rabies and rabies-related virus. *Vaccine* **6,** 533-539.

Fekadu, M., Sumner, J., Shaddock, J., Sanderlin, D., and Baer, G. (1992). Sickness and recovery of dogs challenged with street rabies virus after vaccination with vaccinia virus recombinant expressing rabies virus N protein. *J. Virol.* **66,** 2601-2604.

Flamand, A., Wiktor, T., and Koprowski, H. (1980). Use of hybrydoma monoclonal antibodies in the detection of antigenic difference between rabies and rabies-related virus proteins. I. The nucleocapsid protein. *J. Gen. Virol.* **48,** 97-104.

Fraser, C. F., Hooper, P. T., Lunt, R. A., Gould, A. R., Gleeson, L. J., Hyatt, A. D., Russell, G. M., and Kattenbelt, J. A. (1996). Encephalitis caused by a lyssavirus in fruit bats in Australia. *Emerging Infectious Diseases* **2,** 327-331.

Fredericksen, B. L., and Whitt, M. A. (1996). Mutations at two conserved acidic amino acids in the glycoprotein of vesicular stomatitis virus affect pH-dependent conformational changes and reduce the pH threshold for membrane fusion. *Virology* **217,** 49-57.

Fu, Z. F., Dietzschold, B., Schumacher, C. L., Wunner, W. H., Ertl, H. C. J., and Koprowski, H. (1991). Rabies virus nucleoprotein expressed in and purified from insect cells is efficacious as a vaccine. *Proc Natl Acad Sci USA* **88,** 2001-2005.

Fu, Z., Wunner, W., and Dietzschold, B. (1994). Immunoprotection by rabies virus nucleoprotein. *In "Lyssaviruses"* (C. E. Ruprecht, B. Dietzschold and H. Koprowski Eds.) 161-172. Springer-Verlag, Berlin, Heidelberg, New York

Fu, Z. (1997). Rabies and Rabies research: past, present and future. *Vaccine* **15,** S20-S24.

Fujii, H., Takita-Sonoda, Y., Mifune, K., Hirai, K., Nishizono, A., and Mannen, K. (1994). Protective efficacy in mice of post-exposure vaccination with vaccinia virus recombinant expressing either rabies virus glycoprotein or nucleoprotein. *J. Gen. Virol.* **75,** 1339-1344.

Fynan, E. F., Webster, R. H., Fuller, D. H., Haynes, J. R., Santoro, J. C., and Robinson, H. L. (1993). DNA vaccines: protective immunizations by parenteral, mucosal, and gene-gun inoculations. *Proc. Natl. Acad. Sci. USA* **90,** 11478-11482.

Fynan, E. F., Webster, R. G., Fuller, D. H., Haynes, J. R., Santoro, J. C., and Robinson, H. L. (1995). DNA vaccines: a novel approach to immunization. *Int. J. Immunopharma.* **17,** 79-83.

Gao, L., Barnfield, C. M., Parker, S., Ward, R., and Klavinskis, L. S. (1997). Mucosal delivery of nucleic acid elicits both cellular and humoral immunity. *Biochem. Soc. Trans.* **25,** 338.

Gaudin, Y., Ruigrok, R. W., Tuffereau, C., Knossov, M., and Flamand, A. (1992). Rabies virus glycoprotein is a trimer. *Virology* **187,** 627-632.

Gaudin, Y., Ruigrok, R. W., and Brunner, J. (1993). Low-pH conformational changes of rabies virus glycoprotein and their role in membrane fusion. *J. Virol.* **67,** 1365-1372.

Gaudin, Y., Tuffereau, C., Durrer, P., Flamand, A., and Ruigrock, R. (1995). Biological function of the low-pH, fusion-inactive conformation of rabies virus glycoprotein (G): G is transported in a fusion-inactive state-like conformation. *J. Virol.* **69,** 5528-5533.

Gaudin, Y., Raux, H., Flamand, A., and Ruigrok, R. (1996). Identification of amino acids controlling the low-pH-induced conformational change of rabies virus glycoprotein. *J. Virol.* **70,** 7371-7378.

Gaudin, Y. (1997). Folding of rabies virus glycoprotein: epitope acquisition and interaction with endoplasmic reticulum chaperons. *J. Virol.* **71,** 3742-3750.

Gillis, S., Ferm, M., Ou, W., and Smith, K. (1978). T cell growth factor: parameters of production and a quantitative microassay for activity. *J. Immunol.* **120,** 2027-2032.

Grauballe, P. C., Baagoe, H. M., Fekadu, M., Westergaard, J. M., and Zoffmann, H. (1987). Bat-rabies in Denmark. *Lancet* **1,** 379-80.

Halonen, P. E., Murphy, F. A., Fields, B. N., and Reese, D. R. (1968). Hemagglutination of rabies and some other bullet-shaped viruses. *Proc. Soc. Exp. Biol. Med.* **127,** 1037-1042.

Hassett, D., and Whitton, L. (1996). DNA immunization. *Trends in Microbiol.* **4,** 307-312.

Hemachudha, T. (1994). Human rabies: clinical aspects, pathogenesis and potential therapy. *In "Lyssaviruses* " (C. E. Ruprecht, B. Dietzschold and H. Koprowski Eds), 121-144. Springer-Verlag, Berlin, Heidelberg, New York

Herzog, M., Fritzell, C., Lafage, M., Montano-Hirose, J., and Scott-algara, D. (1991). T and B cell human responses to European bat lyssavirus after post-exposure rabies vaccination. *Clin. exp. Immunol.* **85,** 224-230.

Hofland, H., Shephard, L., and Sullivan, S. M. (1996). Formation of stable cationic lipid/DNA complexes for gene transfer. *Proc. Natl. Acad. Sci. USA* **14**, 7305-7309

Hohlfeld, R., and Engel, A. G. (1994). The immunobiology of muscle. *Immunol.Today* **15,** 269-274.

Hooper, C., Pierard, I., Modelska, A., Otvos, L., Fu, Z., Koprowski, H., and Dietzschold, B. (1994). Rabies nucleocapsid as an oral immunogen and immunological enhancer. *Proc. Natl. Acad. Sci. USA.* **91,** 10908-10912.

Hu, G. (1993). DNA polymerase-catalyzed addition of nontemplated extra nucleotides to the 3' end of a DNA fragment. *DNA Cell Biol.* **12,** 763-770.

Huber, B. T. (1992). Mls genes and self-superantiges. *Trends Genet.* **8,** 399.

Hummeler, K., Koprowski, H., and Wiktor, T. (1967). Structure and development of rabies virus in tissue culture. *J. Virol.* **1,** 152-170.

Hurtley, S. M., and Helenius, A. (1989). Protein oligomerization in the endoplasmatic reticulum. *Annu. Rev. Cell. Biol.* **5,** 277-307.

Hwang, C., Sinskey, A. H., and Lodish, H. F. (1992). Oxidized redox state of glutathione in the endoplasmic reticulum. *Science* **257,** 1496-1502.

Janeway, C. A. (1991). Immune recognition: Mls- makes a little sense. *Nature (Lond).* **349,** 459.

Janeway, C. A. J., and Travers, P. (1996). *Immunobiology: the immune system in health and disease.* Part IV (C. A. J. Janeway and P. Travers Eds), Current Biology Ltd., Middlesex, Cleveland Street, London, and Garland Publishing Inc., New York

Joffret, M. L., Zanetti, C., Morgeaux, S., Leclerc, C., Sureau, P., and Perrin, P. (1991). Appraisal of rabies vaccine potency by determination of *in vitro,* specific interleukin-2 production. *Biologicals* **19,** 113-123.

Kappler, J. W., Wade T., White J., Kushnir E., Blackman M., Bill J., Roehm N., and Marrack P. (1987). A T cell receptor Vb segment that imparts reactivity to a class II major histocompatibility complex product. *Cell* **49,** 263-271

Karpati, G., Pouliot, Y., and Carpenter, S. (1988). Expression of immunoreactive major histocompatiblity complex products in human skeletal muscles. *Ann. Neurol.* **23,** 64-72.

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

Kissi, B., Tordo, N., and Bourhy, H. (1995). Genetic polymorphism in the rabies virus nucleoprotein gene. *Virology* **209,** 526-537.

Klepfer S. R., D. C., Uffelman J., Jacobs P., Bollen A., and Jones E. V. (1993). Characterization of rabies glycoprotein expressed in yeast. *Arch. Virol.* **128,** 269-286.

Koprowski, H., Mocarelli, P., and Wiktor, T. (1972). Antibody response *in vitro* to an animal virus: production of rabies virus neutralizing antibodies by mouse cells in culture. *Proc. Natl. Acad. Sci. USA.* **69,** 2433-2436.

Koprowski, H., Wiktor, T., and Abelseth, M. (1985). Cross-reactivity and cross-protection: rabies variants and rabies-related viruses. *In "Rabies in the topics* (E. Kuwert Ed.), 30-39. Springer-Verlag, Berlin.

Kuroda, K., Geyer, H., Geyer, R., Doerfler, W., and Klenk, H.-D. (1990). The oligosaccharides of influenza virus hemagglutinin expressed in insect cells by a baculovirus vector. *Virology* **174,** 418-429.

Lafay, F., Benmansour, A., Chebli, K., and Flamand, A. (1996). Immunodominant epitopes defined by a yeast-expressed library of random fragments of the rabies virus glycoprotein map outside major antigenic sites. *J. Gen. Virol.* **77,** 339-346.

Lafon, M., Wiktor, T., and MacFarlan, R. (1983). Antigenic sites on the CVS rabies virus glycoprotein: analysis with monoclonal antibodies. *J. Gen. Virol.* **64,** 843-845.

Lafon, M., Ideler, J., and Wunner, W. (1984). Investigation of the antigenic structure of rabies virus glycoprotein by monoclonal antibodies. *Develop. Biol. Standard.* **57,** 219-225.

Lafon, M., Perrin, P., Versmisse, P., and Sureau, P. (1985a). Use of a monoclonal antibody for quantitation of rabies vaccine glycoprotein by enzyme immunoassay. *J. Biol. Stand.* **13,** 295-301.

Lafon, M., and Wiktor, T. (1985b). Antigenic sites on the ERA rabies virus nucleoprotein and non-structural protein. *J. Gen. Virol.* **66,** 2125-2133.

Lafon, M., Herzog, M., and Sureau, P. (1986). Human rabies vaccines induce neutralizing antibodies against the European Bat rabies virus (Duvenhage). *Lancet* 515.

Lafon, M., and Lafage, M. (1987). Antiviral activity of monoclonal antibodies specific for the internal protein N and NS of rabies virus. *J. Gen. Virol.* **68,** 3113-3123.

Lafon, M., Bourhy, H., and Sureau, P. (1988). Immunity against the European bat rabies (Duvenhage) virus induced by rabies vaccines: an experimental study in mice. *Vaccine* **6,** 362-368.

Lafon, M., Lafage, M., Martinezarends, A., Ramirez, R., Vuillier, F., Charron, D., Lotteau, V., and Scottalgara, D. (1992). Evidence for a Viral Superantigen in Humans. *Nature* **358,** 507-510.

Lafon, M. (1993). Rabies virus superantigen. *Res. in Immunol.* 209/213.

Lafon, M., Scott-Algara, D., Marche, P., Cazenave, P.-A., and Jouvin-Marche, E. (1994). Neonatal deletion and selective expension of mouse T cells by exposure to rabies virus nucleocapsid superantigen. *J. Exp. Med.* **180,**

Lafon M.and Galelli, A. (1996). Superantigen related to rabies. *Springer Seminars in Immopathology* **17,** 307 - 318.

Lai, C. Y., and Dietzschold, B. (1981). Amino acid composition and terminal sequence analysis of the rabies virus glycoprotein: identification of the reading frame on the cDNA sequence. *Biochem. Biophys. Res. Comm.* **103,** 536-542.

Larson, J., Wunner, W., Otvos, L., and Ertl, H. (1991). Identification of an immunodominant epitope within the phosphoprotein of rabies virus that is recognized by both class I- and class II-restricted T cells. *J. Virol.* **65,** 5673-5679.

Larson, J., Wunner, W., and Ertl, H. (1992). Immune response to the nominal phosphoprotein of rabies virus. *Virus Research* **23,** 73-88.

Lathe, R. F., Kieny, M. P., Schmitt, D., Curtis, P., and Lecocq, J. P. (1984). M13 bacteriophage vectors for the expression of foreign proteins in Escherichia coli: the rabies glycoprotein. *J. Mol. Appl. Gen.* **2,** 331-342.

Leclerc, C., Dériaud, E., Rojas, M., and Whalen, R. (1997). The preferential induction of Th1 immune response by DNA-based immunization is mediated by immunostimulatory effect of plasmid DNA. *Cell. Immunol.* **179,** 97-106.

Lefrancois, L. (1984). Protection against lethal viral infection by neutralizing and nonneutralizing monoclonal antibodies: distinct mechanisms of action. *J. Virol.* **51,** 208-214.

Lentz, T., Burrage, T. G., Smith, A. L., Crick, J., and Tignor, G. H. (1981). Is the acetylcholine receptor a rabies virus receptor? *Science* **215,** 182-184.

Libeau, G., Lafon, M., and Rollin, P. (1984). Etude de la spécificité d'anticorps monoclonaux obtenus avec la souche de virus de rage Pasteur PV. *Rev. Elev. Med. Vét. Pays Trop.* **37,** 383-394.

Lin, H., Parmacek, M. S., Morle, G., Bolling, S., and Leiden, J. M. (1990). Expression of recombinant genes in myocardium *in vivo* after direct injection of DNA. *Circulation* **82,** 2217-21.

Lin, H., Gontier, C., Saron, M.-F., and Perrin, P. (1993). A new immunostimulatory complex (PICKCa) in experimental rabies: antiviral and adjuvant effects. *Arch. Virol.* **131,** 307-319.

Lodmell, D., and Ewalt, L. (1987). Immune sera and antiglycoprotein monoclonal antibodies inhibit in vitro cell-to-cell spread of pathogenic rabies viruses. *J. Virol.* **61,** 3314-3318.

Lodmell, D., Wiedbrauk, D., and Ewalt, L. (1989). Interferon induced within central nervous system during infection is inconsequential as mechanism responsible for murine resistance to street rabies virus. *J. Gen. Virol.* **70,** 473-478.

Lodmell, D., Sumner, J., Esposito, J., Bellini, W., and Ewalt, L. (1991). Racoon poxvirus recombinants expressing the rabies virus nucleoprotein protect mice against lethal rabies virus infection. *J. Virol.* **65,** 3400-3405.

Lodmell, D., Esposito, J., and Ewalt, L. (1993). Rabies virus antinucleoprotein antibody protect against rabies virus challenge *in vivo* and inhibits rabies virus replication in vitro. *J. Virol.* **67,** 6080-6086.

Luckow, V. A., and Summers, M. D. (1988). Trends in the development of baculovirus expression vectors. *Biotechnology* **6,** 47-55.

Lumio, J., Hillborn, M., Roine, R., Ketonen, L., Haltia, M., Valle, M., Neuvonen, E., and Lahdevirta, M. (1986). Human rabies of bat origin in Europe. *The Lancet* **1,** 378.

MacFarlan, R., Dietzschold, B., Wiktor, T., Kiel, M., Houghten, R., Lerner, R., Sutcliffe, G., and Koprowski, H. (1984). T cell responses to cleaved rabies virus glycoprotein and to synthetic peptides. *J. Immunol.* **133,** 2748-2752.

MacFarlan, R., Dietzschold, B., and Koprowski, H. (1986). Stimulation of cytotoxic T-lymphocyte response by rabies virus glycoprotein and identification of an immunodominant domain. *Mol. Immunol.* **23,** 733-741.

MacPherson, I. and Stocker, M. (1962). Polyoma transformation of hamster cell clones- an investigation of genetic factors affecting cell competence. *Virology* **16**, 147-153

Maiorella, B., Inlow, D., Shauger, A., and Harano, D. (1988). Large-scale insect cell-culture for recombinant protein production. *Bio/Technology* **6,** 1406-1410.

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982). "Molecular Cloning" A Laboratory Manual. (T. Maniatis Ed.) Cold Spring Harbour Laboratory, New York

Manickan, E., Rouse, R. J., Yu, Z., Wire, W. S., and Rouse, B. T. (1995). Genetic immunization against herpes simplex virus. Protection is mediated by CD4+ T lymphocytes. *J. Immunol.* **155,** 259-265.

Marrack, P., and Kappler, J. (1990). The staphyloccal enterotoxins and their relatives. *Science* **248,** 685-686.

Matsuura, Y., Possee, R. D., Overton, H. A., and Bishop, D. H. L. (1987). Baculovirus expression vectors: the requirements for high level expression of proteins, including glycoproteins. *J. Gen. Virol.* **68,** 1233-1250.

McDonnell, M., and Askari, F. (1996). Molecular medicine: DNA vaccine. *New England J. Med.* **334,** 42-45.

McGarvey, B., Hammond, J., Dienelt, M. M., Hooper, D. C., Fu, Z. F., Dietzschold, B., Koprowski, H., and Michaels, F. (1995). Expression of the rabies virus glycoprotein in transgenic tomatoes. *Biotechnology* **13,** 1484-1489.

Meslin, F.-X., Fishbein, D., and Matter, H. (1994). Rationale and prospects for rabies elimination in developing countries. *In "Lyssaviruses"* (C. E. Ruprecht, B. Dietzschold and H. Koprowski Eds.), 1-26. Springer-Verlag, Berlin, Heidelberg, New York

Mifune, K., Schichijo, A., Makino, Y., Takeuchi, E., Yamada, A., and Sakamoto, K. (1980). A mouse model for the pathogenesis and postexposure prophylaxis of rabies. *Microbiol. Immunol.* **24,** 835-845.

Mifune, K., Takeuchi, E., Napiorkowski, P. A., Yamada, A., and Sakamoto, K. (1981). Essential role of T-cells in the post-exposure prophylaxis of rabies in mice. *Microbiol. Immunol.* **25,** 895-904.

Mollgaard, S. (1985). Bat-rabies in Denmark. *Rabies Bulletin Europe* **4,** 11-13.

Morimoto K., O. A., and Kawai A. (1989). Structure and transcription of the glycoprotein gene of attenuated HEP-Flury strain of rabies virus. *Virology* **173,** 465-477.

Morimoto, K., Ni, Y.-J., and Kawai, A. (1992). Syncytium formation is induced in the murine neuroblastoma cell cultures which produce pathogenic type G proteins of the rabies virus. *Virology* **189,** 203-216.

Mosmann, T. R., and Coffman, R. L. (1989). TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Ann. Rev. Immunol.* **7,** 145-173.

Mougin B., G. P., Bakouche O., and Wild T. F. (1987). Measles virus subunit vaccine: importance of viral glycoproteins presentation. *In "The Biology of Negative Strand Viruses* (Kolakofsky M. Ed.), 316-319, New York, USA.

Murhammer, D. W., and Goochee, C. F. (1988). Large scale-production of insect cell cultures: protective effect of Pluronic F-68. *Bio/Technology* **6,** 1411-1418.

Murphy, F., Harrison, A., Winn, W., and Bauer, S. (1973). Comparative pathogenesis of rabies and rabies-like viruses. Infection of the central nervous system and centrifugal spread of virus to peripheral tissues. *Lab. Invest.* **29,** 1-16.

Murphy, F. A., and Bauer, S. P. (1974). Early street rabies virus infection in striated muscle and later progression to the central nervous system. *Intervirology* **3,** 256-268.

Murphy, F. A. (1977). Rabies pathogenesis. *Arch. Virol.* **54,** 270-297.

Nabel, E. G., Plautz, G., and Nabel, G. J. (1990). Site-specific gene expression in vitro by direct gene transfer into the arterial wall. *Science* **249,** 1285-1288.

Nunberg, J., Doyle, M., York, S., and York, C. (1989). Interleukin-2 acts as an adjuvant to increase the potency of inactivated rabies virus vaccine. *Proc. Natl. Acad. Sci. USA* **86,** 40-42.

Oth, D., Mercier, G., Perrin, P., Joffret, M.-L., Sureau, P., and Thibodeau, L. (1987). The association of the rabies glycoprotein with liposome (immunosome) induces an in vitro specific release of Interleukin-2. *Cell. Immunol.* **108,** 220-226.

Paoletti, E., Lipinskas, B. F., Samsonoff, C., Mercer, S., and Panicali, D. (1984). Construction of live vaccines using genetically engineered poxviruses: biological activity of vaccinia virus recombinants expressing the hepatitis B virus surface antigen and the herpes simplex virus glycoprotein D. *Proc. Natl. Acad. Sci. USA* **81,** 193-197.

Parry, N., Fox, G., Rowlands, C., Brown, F., Fry, E., Acharya, R., Logan, D., and Stuart, D. (1990). Structural and serological evidence for a novel mechanism of antigenic variation in foot-and-mouth disease virus. *Nature* **347,** 569-572.

Pasteur, L. (1885). Méthode pour prévenir la rage après morsure. *Compt. Rendus Acad. Sci., Paris* **17,** 765-774.

Pereira, C., Nozaki-Renard, J., Schwartz, J., Eyquem, A., and Atanasiu, P. (1982). Cytotoxicity reactions against target cells infected with rabies virus. *J. Virol. Meth.* **5,** 75-83.

Perrin, P., and Atanasiu, P. (1980). Partial structural study of oligosides of rabies virus glycoprotein. *Microbiologica.* **3,** 57-74.

Perrin, P., and Atanasiu, P. (1981). Rabies glycoprotein affinity for lipids. I. Structural study of different types of spontaneous and induced associations. *Microbiologica* **4,** 231-247.

Perrin, P., Portnoi, D., and Sureau, P. (1982). Etude de l'adsorption et de la pénétration du virus rabique: interactions avec les cellules BHK-21 et des membranes artificielles. *Ann. Virol. (Inst. Pasteur)* **133E,** 403-422.

Perrin, P., Thibodeau, L., Dauguet, C., Fritsch, A., and Sureau, P. (1984). Amplification des propriétés immunogènes de la glycoprotéine rabique par ancrage sur des liposomes préformés. *Ann. Virol. (Inst. Pasteur)* **135E,** 183-199.

Perrin, P., Lafon, M., Versmisse, P., and Sureau, P. (1985a). Application d'une méthode immunoenzymatique au titrage des anticorps rabiques en culture cellulaire. *J. Biol. Standard.* **13,** 35-42.

Perrin, P., Sureau, P., and Thibodeau, L. (1985b). Rabies immunosomes efficiency in pre- and post-exposure vaccinations: role of liposomes in glycoprotein conformation and immunogenicity. *In "Synthetic immunomodulators and vaccines"* (Zaoral, M. et al. Eds) 252-271. Prague, Czechoslovakia

Perrin, P., Thibodeau, L., and Sureau, P. (1985c). Rabies immunosomes (subunit vaccine) structure and immunogenicity. Pre- and post exposure protection studies. *Vaccine* **3,** 325-332.

Perrin, P., Rollin, P., and Sureau, P. (1986a). A rapid rabies enzyme immuno-diagnosis (RREID): a useful and simple technique for the routine diagnosis of rabies. *J. Biol. Standard.* **14,** 217-222.

Perrin, P., Versmisse, P., Delagneau, J. F., Lucas, G., Rollin, P., and Sureau, P. (1986b). The influence of the type of immunosorbent on rabies antibody EIA ; advantages of purified glycoprotein over whole virus. *J. Biol. Standard.* **14,** 95.

Perrin, P., Joffret, M.-L., Leclerc, C., Oth, D., Sureau, P., and Thibodeau, L. (1988a). Interleukin-2 increases protection against experimental rabies. *Immunobiol.* **177,** 199.

Perrin, P., Joffret, M.-L., Oth, D., Leclerc, C., Sureau, P., and Thibodeau, L. (1988b). Interleukin-2 production in vitro : a new approach to the study of rabies vaccine immunogenicity as appraised by testing different glycoprotein presentations. *Vaccine* **6,** 331.

Perrin, P., Joffret, M.-L., Morgeaux, S., Leclerc, C., Oth, D., Thibodeau, L., and Sureau, P. (1989). Role of interleukin-2 in antirabies immune response. *In"Advances in animal cell biology and technology for bioprocesses"* (Spier, R. et al. Eds.), 494-496. Butterworth & Co., Great Britain

Perrin, P., Lafon, L., and Sureau, P. (1990). Rabies vaccines from Pasteur's time up to experimental subunit vaccines Today. *In "Viral Vaccines"* , 325-345. Wiley-Liss, Inc., New York, Chichester, Brisbane

Perrin, P. (1996a). *In "Laboratory techniques in rabies"* (F.-X. Meslin, M. M. Kaplan and H. Koprowski Ed.), 433-457, WHO, Geneva

Perrin, P., Tino de Franco, M., Jallet, C., Fouque, F., Morgeaux, S., Tordo, N., and Colle, J.-H. (1996b). The antigen-specific cell-mediated immune response in mice is suppressed by infection with pathogenic Lyssaviruses. *Res. Virol.* **147,** 289-299.

Perry, L. L., and Lodmell, D. L. (1991). Role of CD4+ and CD8+ T-Cells in Murine Resistance to Street Rabies Virus. *J. Virol* **65,** 3429-3434.

Pinto, R., Bosh, A., and Bishop, D. (1994). Structures associated with the expression of rabies virus stuctural genes in insect cells. *Virus Res.* **31,** 139-145.

Plotkin, S. (1993). Vaccination in the 21st Century. *J. Infect. Dis.* **168,** 29-37.

Prabhakar, B. S., and Nathanson, N. (1981). Acute rabies death mediated by antibody. *Nature* **290,** 590-591.

Prehaud, C., Coulon, P., Lafay, F., Thiers, C., and Flamand, A. (1988). Antigenic site II of the rabies virus glycoprotein: structure and role in viral virulence. *J. Virol.* **62,** 1-7.

Prehaud, C., Takehara, K., Flamand, A., and Bishop, D. H. L. (1989). Immunogenic and protective properties of rabies virus glycoprotein expressed by baculovirus vectors. *Virology* **173,** 390-399.

Prevec, L., Campbell, J. B., Christie, B. S., Belbeck, L., and Graham, F. L. (1990). A recombinant human adenovirus vaccine against rabies. *J. Inf. Dis.* **161,** 27.

Raz, E., Carson, D. A., Parker, S. E., Par, T. B., Abai, A. M., Aichinger, G., Gromkowski, S. H., Singh, M., Lew, D., Yankauckas, M. A., et al. (1994). Intradermal gene immunization: the possible role of DNA uptake in the induction of cellular immunity to viruses. *Proc. Natl. Acad. Sci. USA* **91,** 9519-9523.

Roitt, I. M., Brostoff, J., and Male, D. K. (1993). *In* "Immunology", (I. M. Roitt, J. Brostoff and D. K. Male Eds), chapter 7, 3rd edition, Mosby, St. Louis, Baltimore, Boston

Rose, J. K., Doolittle, R. F., Anilionis, A., Curtis, P. J., and Wunner, W. H. (1982). Homology between the glycoproteins of vesicular stomatitis virus and rabies virus. *J. Virol.* **43,** 361-364.

Rötzschke, O., and Falk, K. (1991). Naturally-occurring peptide antigens derived from the MHC class-I-restricted processing pathway. *Immunol. Today* **12,** 447-455.

Russell, S. M., and Liew, F. Y. (1979). T cells primed by influenza virion internal components can cooperate in the antibody response to hemagglutin. *Nature* **280,** 147-148.

Sato, Y., Roman, M., Tighe, H., Lee, D., Corr, M., Nguyen, M.-D., Silvermann, G. J., Lotz, M., Carson, D. A., and Raz, E. (1996). Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science* **273,** 352-354.

Schijns, V., Classen, I., Vermeulen, A., Horzinek, M., and Osterhaus, A. (1994). Modulation of antiviral immune responses by exogenous cytokines: effects of tumour necrosis factor-α, interleukin-1a, interleukin-2 and interferon-γ on the immunogenicity of an inactivated rabies vaccine. *J. Gen. Virol.* **75,** 55-63.

Schneider, L., Dietzschold, B., Dierks, R., Mathaeus, W., Enzman, P., and Strohmaier, K. (1973). Rabies group-specific ribonucleoprotein antigen and test system for grouping and typing rhabdoviruses. *J. Virol.* **11,** 748-755.

Schneider, L. G. (1982). Antigenic variants of rabies virus. *Comp. Imm. Microbiol. Inf. Dis.* **5,** 101-107.

Schumacher C. L., D. B., Ertl H. C. J., Niu H.-S., Rupprecht C. E., and Koprowski H. (1989). Use of mouse anti-rabies monoclonal antibodies in postexposure treatment of rabies. *J. Clin. Invest.* **84,** 971-975.

Schumacher, C. L., Ertl, H. C. J., Koprowski, H., and Dietzschold, B. (1992). Inhibition of immune responses against rabies virus by monoclonal antibodies directed against rabies virus antigens. *Vaccine* **10,** 754-760.

Sedegah, M., Hedstrom, R., Hobart, P., and Hoffmann, S. L. (1994). Protection against malaria by immunization with plasmid DNA encoding circumsporozoite protein. *Proc. Natl. Acad. Sci. USA* **91,** 9866-9870.

Seif, I., Coulon, P., Rollin, P., and Flamand, A. (1985). Rabies virulence: effect on pathogenicity and sequence characterization of rabies virus mutations affecting antigenic site III of the glycoprotein. *J. Virol.* **53,** 926-934.

Selimov, M. A., Tatarov, A. G., Botvinkin, A. D., Klueva, E. V., Kulikova, L. G., and Khismatullina, N. A. (1991). Rabies-related Yuli virus: identification with a panel of monoclonal antibodies. *Acta virol* . **33,** 542-546.

Shankar, V., Dietzschold, B., and Koprowski, H. (1991). Direct entry of rabies virus into the central nervous system without prior local replication. *J. Virol.* **65,** 2736-2738.

Sikes, R. K., Cleary, W. F., Koprowski, H., Wiktor, T. J., and Kaplan, M. M. (1971). Effective protection of monkeys against death from street virus by post-exposure administration of tissue culture rabies vaccine. *Bull. Wld. Hlth. Org.* **45,** 1-11.

Smith, G. E., Summers, M. D., and Fraser, M. J. (1983). Production of human beta interferon in insect cells infected with a baculovirus expression vector. *Mol. Cell. Biol.* **3,** 2156-2165.

Smith, G. E., Ju, G., Ericson, B. L., Moschera, J., Lahm, H., Chizzonite, R., and Summers, M. D. (1985). Modification and secretion of human interleukin 2 produced in insect cells by a baculovirus expression vector. *Proc. Nat. Acad. Sci. USA* **82,** 8404-8408.

Smith, G. L., Machett, M., and Moss, B. (1983a). Infectious vaccinia virus recombinants that express hepatitis B virus surface antigen. *Nature* **302,** 490-495.

Smith, G. L., Murphy, B. R., and Moss, B. (1983b). Construction and characterization of an infectious vaccinia virus recombinant that expresses the influenza hemagglutinin gene and induces resistance to influenza virus infection in hamsters. *Proc. Natl. Acad. Sci. USA* **80,** 7155-7159.

Smith, J., Yager, P., and Baer, G. (1973). A rapid reproducible test for determining rabies neutralizing antibody. *Bull. Wld. Hlth. Org.* **48,** 535-541.

Smith P. K., Krohn, R. I., Hermanson, G. T., Mallia, A., K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150,** 76-85.

Sokol, F., Schlumberger, D., Wiktor, T., and Koprowski, H. (1969). Biochemical and biophysical studies on the nucleocapsid and on the RNA of rabies virus. *Virology* **38,** 651-665.

Sokol, F. (1973a). Purification of rabies virus and isolation of its components. *In "Laboratory techniques in rabies". WHO, Geneva.* 165-178.

Sokol, F., and Clark, F. (1973b). Phosphoproteins, structural components of rhabdovirus. *Virology* **52,** 246-263.

Summers, M. D., and Smith, G. E. (1988). A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures. Texas Agricultural Experiment Station, No. 1555

Sumner, J., Fekadu, M., Shaddock, J., Esposito, J., and Bellini, W. (1991). Protection of mice with vaccinia virus recombinants that express the rabies nucleoprotein. *Virology* **183,** 703-710.

Superti, F., Derer, M., and Tsiang, H. (1984). Mechanism of rabies virus entry into CER cells. *J. Gen. Virol.* **65,** 781-789.

Superti, F., Hauttecoeur, B., Morelec, M.-J., Goldoni, P., Bizzini, B., and Tsiang, H. (1986). Involvement of gangliosides in rabies virus infection. *J. Gen. Virol.* **67,** 47-56.

Sureau, P. (1988). New vaccines for immunization of man: new approaches towards the prevention of rabies in man. *Parasitologia* **30,** 141-148.

Sureau, P. (1992). Contribution to rabies prevention. *Vaccine* **10,** 896-899.

Süss, J., and Sinnecker, H. (1991). Immune reactions against rabies viruses-infection and vaccination. *Exp. Pathol.* **42,** 1-9.

Takita-Sonada, Y., Fujii, H., Mifune, K., Ito, Y., Hiraga, M., Nishizono, A., Mannen, K., and Minamoto, N. (1993). Resistance of mice vaccinated with rabies virus internal structural proteins to lethal infection. *Arch. Virol.* **132,** 51-65.

Tang, D., DeVit, M., and Johnston, S. A. (1992). Genetic immunization is a simple method for eliciting an immune response. *Nature* **356,** 152-154.

Taylor, J., Meigneir, B., Tartaglia, J., Languet, B., VanderHoeven, J., Franchini, G., Trimarchi, C., and Paoletti, E. (1995). Biological and immunogenic properties of a canary-rabies recombinant, ALVAC-RG (vCP65) in non avian species. *Vaccine* **13,** 539-549.

Tollis, M., Dietzschold, B., Buona Volia, C., and Koprowski, H. (1991). Immunization of monkeys with rabies ribonucleoprotein (RNP) confers protective immunity against rabies. *Vaccine* **9,** 134-136.

Tordo, N., Poch, O., Ermine, A., and Keith, G. (1986a). Primary structure of leader RNA and nucleoprotein genes of the rabies genome: segmented homology with VSV. *Nucl. Acids. Res.* **14,** 2671-2683.

Tordo, N., Poch, O., Ermine, A., Keith, G., and Rougeon, F. (1986b). Walking along the rabies genome: Is the large G-L intergenic region a reminant gene? *Proc. Natl. Acad. Sci. USA* **83,** 3914-3918.

Tordo, N., and Poch, O. (1988). Strong and weak transcription signals within the rabies genome. *Virus Res.* **sup. 2,** 30-35.

Tordo, N., Bourhy, H., Sather, S., and Ollo, R. (1993). Structure and expression in baculovirus of the Mokola virus glycoprotein: an efficient recombinant vaccine. *Virology* **194,** 59-69.

Tsiang, H. (1993). Pathophysiology of rabies virus infection of the nervous system. *Adv. Virus Research.* **42,** 375-412.

Tuchiya, K., Matsuura, Y., Kawai, A., Ishihama, A., and Ueda, S. (1992). Characterization of rabies virus glycoprotein expressed by recombinant baculoviruses. *Virus Res.* **25,** 1-13.

Tuffereau, C., Leblois, H., Benejean, J., Coulon, P., Lafay, F., and Flamand, A. (1989). Arginine or lysine in position 333 of ERA and CVS glycoprotein is necessary for rabies virulence in adult mice. *Virology* **172,** 206-212.

Turner, G. (1976a). Thymus dependence of rabies vaccine. *J. Gen. Virol.* **33,** 535-538.

Turner, G., and Ballard, R. (1976b). Interaction of mouse peritoneal macrophages with fixed rabies virus *in vivo* and *in vitro*. *J. Gen. Virol.* **30,** 223-231.

Turner, G. (1978). Immunoglobulin (IgG) and (IgM) responses to rabies vaccine. *J. Gen. Virol.* **40,** 595-604.

Ulmer, J. B., Donnelly, J. J., Parker, S. E., Rhodes, G. H., Felgner, P. L., Dwarki, V. J., Gromkowski, S. H., Deck, R. R., DeWitt, C. M., Friedmann, A., Hawe, L. A., Leander, K. R., Martinez, D., Perry, H. C., Shiver, J. W., Montgomery, D. L., and Liu, M. A. (1993). Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* **259,** 1745-1749.

Unanue, E. (1993). Macrophages, antigen-presenting cells, and the phenomena of antigen handling and presentation. *In "Fundamental Immunology"* 111-144 (Paul, W. E. Ed.), Raven Press, New York

van Rooijen, N. (1990). Liposomes as carrier and immunoadjuvant of vaccine antigens. *In "Bacterial Vaccines"* 255-279 (Ed. Alan R.) Liss Inc.,

Vishwanath, R. L., Katz, F. N., Lodish, H. F., and Blobel, G. (1978). A signal sequence for the insertion of a transmembrane glycoprotein. *J. Biol. Chem.* **253,** 8667-8670.

Wang, B., Ugen, K. E., Srikantan, M. G., Dang, K., Refaeli, Y., Sato, A. I., Boyer, J., Williams, W. V., and Weiner, D. B. (1993). Gene inoculation generates immune responses against human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. USA* **90,** 4156.

Wang, Y., Xiang, Z., Pasquini, S., and Ertl, H. C. J. (1997). Immune response to neonatal genetic immunization. *Virology* **228,** 278-284.

Weiland, F., Cox, J., Meyer, S., Dahme, E., and Reddehase, M. (1992). Rabies virus Neuritic paralysis: immunopathogenesis of nonfatal paralytic rabies. *J. Virol.* **66,** 5096-5099

Weiss, R. A., and Bennet, P. L. P. (1980). Assembly of membrane glycoproteins studied by phenotypic mixing between mutants of vesicular stomatitis virus and retroviruses. *Virology* **100,** 252-274.

Welsh, R. M., Tay, C. H., Varga, S. M., O'Donnell, C. L., Vergilis, K. L., and Selin, L. K. (1996). Lymphocyte-dependent 'natural' immunity to virus infections mediated by both natural killer cells and memory T cells. *Virology* **7,** 95-102.

Whitby, J. E., Johnstone, P., Parsons, G., King, A. A., and Hutson, A. M. (1996). Ten-year survey of British bats for the existence of rabies. *Veterinary Record* **139,** 491-493.

Whitt, M., Buonocore, L., Prehaud, C., and Rose, J. (1991). Membrane fusion activity, oligomerization, and assembly of the rabies virus glycoprotein. *Virology* **185,** 681-688.

WHO (1994). World survey of rabies N° 30 for the year 1994. (Division of Emerging and Other Communicable Diseases Surveillance and Control) WHO, Geneva

WHO (1996). Projet de directives pour l'assurance de qualité des vaccins à ADN. (Comité d'Experts de la standardisation biologique) WHO, Geneva

Wiktor, T., Lerner, R., and Koprowski, H. (1971). Inhibitory effect of passive antibody on active immunity induced against rabies by vaccination. *Bull. Wld. Hlth. Org.* **45,** 747-753.

Wiktor, T., Gyorgy, E., Schlumberger, D., Sokol, F., and Koprowski, H. (1973). Antigenic properties of rabies virus components. *J. Immunol.* **110,** 269-276.

Wiktor, T., Doherty, P., and Koprowski, H. (1977). In vitro evidence of cell-mediated immunity after exposure of mice to both live and inactivated rabies vaccines. *Proc. Natl. Acad. Sci. USA* **74,** 334-338.

Wiktor, T., and Koprowski, H. (1978). Monoclonal antibodies against rabies virus produced by somatic cell hybridization: detection of antigenic variants. *Proc. Natl. Acad. Sci. USA* **75,** 3938-3942.

Wiktor, T., Flamand, A., and Koprowski, H. (1980). Use of monoclonal antibodies in diagnosis or rabies virus infection and differentiation of rabies-related virus. *J. Virol. Meth.* **1,** 33-46.

Wiktor, T., MacFarlan, R., Reagan, K., Dietzschold, D., Curtis, P., Wunner, W., Kieny, M.-P., Lathe, R., Lecocq, J.-P., Mackett, M., Moss, B., and Koprowski, H. (1984). Protection from rabies by a vaccinia virus recombinant containing the rabies virus glycoprotein gene. *Proc. Natl. Acad. Sci. USA.* **81,** 7194-7198.

Wiktor, T., Plotkin, S., and Koprowski, H. (1988). Rabies vaccine. In "Vaccines", 474-491 (S. Plotkin and E. Mortimer Eds), W. B. Saunders Company, Philadelphia, London, Toronto

Wildy, P. (1971). Classification and Nomenclature of Viruses. First report of the International Committee on Nomenclature of Viruses. *Mono. Virol.* **5,** 1-14.

Williams, R. S., Johnston, S. A., Riedy, M., DeVit, M. J., McElligott, S. G., and Sanford, J. C. (1991). Introduction of foreign genes into tissues of living mice by DNA-coated microprojectiles. *Proc. Natl. Acad. Sci. USA* **88,** 2726-2730.

Winkler, W. G., and Bögel, K. (1992). Control of rabies in wildlife. *Scientific American* , June, 56-62.

Wojczyk, B., Shakin-Eshleman, S. H., Doms, R. W., Zhi-Quan, X., Ertl, H. C. J., Wunner, W. H., and Spitalnik, S. (1995). Stable secretion of a soluble oligomeric form of rabies virus glycoprotein: influence of N-glycan processing on secretion. *Biochemistry* **34,** 2599-2609.

Wolff, J. A., Malone, R. W., Williams, P., Chong, W., Acsadi, G., Jani, A., and Felgner, P. L. (1990). Direct gene transfer into mouse muscle in vivo. *Science* **247,** 1465-1468.

Wolff, J. A., Williams, P., Acsadi, G., Jiao, S., Jani, A., and Chong, W. (1991). Conditions affecting direct gene transfer into rodent muscle *in vivo*. *Biotechniques* **11,** 474-485.

Wolff, J. A., Ludtke, J. J., Acsadi, G., Williams, P., and Jani, A. (1992). Long-term persistence of plasmid DNA and foreign gene expression in mouse muscle. *Hum. Mol. Gen.* **1,** 363-369.

Wunderli, P., Shaddock, J., Schmid, D., Miller, T., and Baer, G. (1991). The protective role of humoral neutralizing antibody in the NIH potency test for rabies vaccines. *Vaccine* **9,** 638-642.

Wunner, W., Dietzschold, B., Macfarlan, R., Smith, C., Golub, E., and Wiktor, T. (1985). Localization of immunogenic domains on the rabies virus glycoprotein. *Ann. Inst. Pasteur.* **136 E,** 353-362.

Wunner, W., Dietzschold, B., Smith, C., Lafon, M., and Golub, E. (1985). Antigenic variants of CVS rabies virus with altered glycosylation sites. *Virology,* **140,** 1-12.

Wunner, W. H., Larson, J. K., Dietzschold, B., and Smith, C. L. (1988). The molecular biology of rabies viruses. *Rev. Infect. Dis.* **10,** 771-783.

Wunner, W. (1991). The chemical composition and molecular structure of rabies viruses. *In: The natural history of rabies"* (2nd edition, Baer. G. Ed.), 31-67. CRC Press, Boston

Xiang, Z., Spitalnik, S., Tran, M., Wunner, W., Cheng, J., and Ertl, H. (1994). Vaccination with a plasmid vector carrying the rabies virus glycoprotein gene induces protective immunity against rabies virus. *Virology,* **199,** 132-140.

Xiang, Z., and Ertl, H. (1995a). Manipulation of the immune response to a plasmid-encoded viral antigen by coinoculation with plasmids expressing cytokines. *Immunity* **2,** 129-135.

Xiang, Z., Kwowles, B., McCarrick, J., and Ertl, H. (1995b). Immune effector mechanisms required for protection to rabies virus. *Virology* **214,** 398-404.

Xiang, Z., Spitalnik,S., Erikson, J., Wojczyk, B., and Ertl, H. (1995c). Immune responses to nucleic acid vaccines to rabies virus. *Virology* **209,** 569-579.

Xiang, Z. Q., He, Z., Wang, Y., and Ertl, H. C. J. (1997). The effect of interferon-γ on genetic immunization. *Vaccine* **15,** 896-898.

Xu, D., and Liew, F. Y. (1994). Genetic vaccination against leishmaniasis. *Vaccine* **12,** 1534-1536.

Yelverton E., N. S., Obijeski J. F., and Goeddel D. V. (1983). Rabies virus glycoprotein analogs: biosynthesis in Escherichia coli. *Science* **219,** 614-620.

Yokoyama, M. H. D. E., Zhang, J., and Whitton, J. L. (1997). DNA immunization can stimulate florid local inflammation, and the antiviral immunity induced varies depending on injection site. *Vaccine* **15,** 553-560.

Zinkernagel, R. M. (1996). Immunology taught by viruses. *Science* **271,** 173-178.

Zinkernagel, R. M. (1997). Protective antibody responses against viruses. *Biol. Chem.* **378,** 725-729.

APPENDIX

APPENDIX III. 1.: Cell culture media and buffers

Cell culture media

• *Earle's solution (20X):*

1360 g NCl, 80 g KCl, 28 g $NaH_2PO_4(H_2O)$, 40 g $MgSO_4(7H_2O)$, 200 g glucose, 40 g $CaCl_2$, H_2O ad 10 l.

• *Tryptose phosphate buffer (TPB):*

20 g Bacto-tryptose "Difco", 2 g Bactro-dextrose, 5 g NaCl, 2.5 g disodium phosphate, H_2O ad 1l.

• *Antibiotics (Ab):*

Gentamycine (Dakota Pharm) used at 40 mg/l.

• *Trypsin-versene solution:*

Trypsin and Versene are mixed volume to volume.

Versene solution 0.02%, pH 7,8:

80 g NaCl, 2 g KCl, 14.5 g Na_2HPO_4 (H_2O), 2 g Versene (EDTA), 10 ml phenolred 1%, H_2O ad 10 l.

• *Trypsin solution 0.25% pH 7.8:*

25 g trypsin Difco (1/250), 80 g NaCl, 14.1 g Na_2HPO_4 (H_2O), 2 g KCl, 10 ml phenolred 1%, H_2O ad10 l.

• *Bicarbonate solution 5.5% pH 10:*

550g $NaHCO_3$, H_2O ad10 l.

• *Amino acid solution (100X):*

105 g L-arginine, 31 g L-histidine, 52 g L-isoleucine, 52 g L-leucine, 58 g L-lysine, 58 g L-phenylalanine, 48 g L-threonine, 46 g L-valine, 15 g L-methionine, 10g L-tryptophane, H_2O ad 10 l.

• *Amino acid solution (1000X):*

24g L-cystidin, 36g L-tyrosin, disolved in 740ml H_2O and 60ml HCl 2N.

• *Vitamin solution (1000X):*

A

1g biotin, 1g cholinchoride, 1g folic acid, H_2O ad 100 ml.

B

2g inositol, 1g nicotinamid, 1g pyridoxal, 0.1g riboflavin.

Mix A and B, for complete disolution add some drops of NaOH 1N.

• *Non-essential amino acids:*

8.9 mg L-alanine, 15 mg L-asparagine H_2O, 13.3 mg L-aspartic acid, 7.5 mg glycine, 14.7 mg L-glutamic acid, 11.5 mg L-proline, 10.5 mg L-serin, H_2O ad 1 l.

• *RPMI medium for washing:*

1 ml glutamine (Gibco; 30 g/l), 4 mg gentamycine (Dakota Pharm), RPMI medium-1640 ad100 ml.

• *RPMI-1640 medium (Gibco):*
200 mg L-arginine, 50 mg L-asparagine H_2O, 20 mg L-aspartic acid, 50 mg L-cystine, 20 mg L-glutamic acid, 10 mg glycine, 15 mg L-histidine, 20 mg L-hydroxyproline, 50 mg L-isoleucine, 50 mg L-leucine, 0.2 mg D-biotine, 0.25 mg D-Ca-pantothenate, 3 mg choline chloride, 1 mg folic acid, 35 mg I-Inositol, 1 mg nicotinamide, 1 mg p-aminobenzoic acid. 1 mg pyridoxine HCl, 0.2 mg riboflavine, 1 mg thiamine HCl, 40 mg L-lysine HCl, 14 mg L-methionine, 15 mg L-phenylalanine, 20 mg L-proline, 30 mg L-serine, 20 mg L-threonine, 5 mg L-tryptophane, 20 mg L-tyrosine, 20 mg L-valine, 0,005 mg Vitamine B12, 100 mg $Ca(NO_3)_24H_2O$, 400 mg KCl, 100 mg $MgSO_47H_2O$, 6 g NaCl, 2 g $NaHCO_3$, 800 mg Na_2HPO_4, 2 g D-glucose, 1 mg glutatione (reduced), 5 mg phenolred, H_2O ad 1 l.

• *Complete RPMI medium:*
10% decomplemented fetal calf serum (FCS; Tech Gen), 1 ml glutamine (Gibco; 30 g/l)
1 ml Hepes* (1M), 1 ml non-essetial aminoacids (100 mM), 1 ml sodium pyruvate (100 mM), 400 µl b-mercaptoethanol (5.10^5M)**, RPMI-1640 medium ad100ml.
*N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (Gibco)
**b-mercaptoethanol 5.10^5 M: dilute 10 µl/10 ml medium, filter (0.22 µm)

• *Eagle's minimum essential medium (MEM)-TPB-Glu:*
500 ml Earle 20X, 7 l H_2O, 100 ml amino acids 100X, 10 ml amino acids 1000X, 10 ml vitamins 1000X, 6 g glutamine, 1 l TPB, 20 ml sodic phenolred 1%, H_2O ad 10 l.

Buffers for ELISA test

• *Phosphate buffer saline 20X (PBS) pH 7.3:*
1600 g NaCl, 40 g KCl, 228 g $Na_2HPO_42H_2O$, 40 g KH_2PO_4, H_2O ad10 l, check pH 7.3 +/- 0.05.

• *Carbonate-bicarbonate buffer pH 9.6:*
$Na_2CO_310H_2O$ 0.05 M, $NaHCO_3$ 0.05 M. For 100 ml $NaHCO_3$ 0.05 M add 10 ml of Na_2CO_3 0.05 M and continue adding to obtain pH 9.6.

• *Stabilisation solution, pH 9.6:*
5 g saccharose, 0.3 g bovine serum albumin (BSA) fraction 5 (Sigma), carbonate-bicarbonate buffer pH 9.6 ad100 ml, adjust pH at 9.6 by adding Na_2CO_3 solution 0.05 M.

• *PBS-Tween buffer, pH 7.4:*
50 ml PBS 20X, 0.5 ml Tween 20 (Sigma), H_2O ad 1000 ml.

• *PBS-Tween-BSA buffer, pH 7.2:*
0.5 g BSA fraction 5 (Sigma), PBS-Tween pH 7.4 ad 100 ml
adjust pH at 7.25 by adding NaOH 4 N.
• *Citrate buffer, pH 5.6:*
2.168 g citric acid $1H_2O$, 11.67 g trisodic citrate $2H_2O$, H_2O ad 1 l.
• *Chromogen substrate:*
30 ml Citrate buffer pH 5.6, 30 µl H_2O_2 30 % (110 vol), 30 µl Orthophenoldiamin tablets (Sigma).

Buffers for antigen preparation
• Na-Tris-buffer (NT) pH 7.6:
7.6 g NaCl, 6.057 g Tris, HCl 2N ad pH 7.6, H_2O ad 1 l.
• *Phosphate Buffer Saline (PBS) pH 7.4:*
8.0 g NaCl, 0.2 g KCl, 2.9 g $Na_2HPO_412H_2O$, 0.2 g KH_2PO_4, H_2O ad 1 l, check pH 7.4.
• *PBS Ca2+ Mg2+ buffer (PBS Ca/Mg):*
0.1 g $MgSO_47H_2O$, 0.076 g $CaCl_2$, PBS ad 1 l.

Bacteria culture media
• *LB (Luria Bertani):*
Liquid medium: 10 g Bacto-tryptone (Difco), 5 g Bacto-yeast extract (Difco), 10 g NaCl, adjust pH at 7.5 with 10N NaOH, H_2O ad1 l.

Solid medium: 10 g Bacto-tryptone, 5 g Bacto-yeast extract, 10 g NaCl, 15g Bacto-agar (Difco), adjust pH at 7.5 with 10N NaOH, H_2O ad1 l.

Autoclave for 30 min at 120°C. Add ampicilline (Totapen, Bristol-Myers Squibb) 0,1 mg/ml after the solution has cooled down.
• *Terrific Broth:*
12 g Bacto-tryptone, 24 g Bacto-yeast, 4 ml glycerol. 900 ml of distilled water are added. After autoclaving, the solution is cooled down to <60°C before adding 100 ml of sterile 10X TB Phosphate (0.17 M KH_2PO_4, 0.72 M K_2HPO_4). Ampicilline 0,1 mg/ml is added.

Appendix III. 2.: Open reading frame of the Mok/PV gene on the pMok/PV plasmid.

1/1 **Signal peptide** 31/11 ->

ATG AAT ATA CCT TGC TTT GTT GTG ATT CTC AGC TTA GCC ACT ACA CAT TCT CTG GGA GAA

M N I P C F V V I L S L A T T H S L G E

61/21 **Ectodomain: site II region Mok** 91/31

TTC CCC TTG TAC ACA ATT CCT GAG AAG ATA GAG AAA TGG ACT CCC ATA GAC ATG ATC CAT

F P L Y T I P E K I E K W T P I D M I H

121/41 151/51

CTG AGT TGC CCC AAC AAC CTA TTA TCT GAG GAA GAA GGT TGC AAT GCA GAG TCA TCC TTT

L S C P N N L L S E E E G C N A E S S F

181/61 211/71

ACT TAC TTT GAG CTC AAG AGT GGT TAC CTA GCT CAT CAG AAG GTT CCA GGG TTT ACC TGT

T Y F E L K S G Y L A H Q K V P G F T C

241/81 271/91

ACC GGG GTC GTG AAC GAG GCA GAG ACA TAT ACA AAC TTC GTC GGG TAC GTC ACC ACA ACC

T G V V N E A E T Y T N F V G Y V T T T

301/101 331/111

TTC AAA AGG AAG CAC TTT AGG CCT ACA GTA GCC GCC TGT CGT GAT GCC TAC AAC TGG AAA

F K R K H F R P T V A A C R D A Y N W K

361/121 391/131

GTG TCA GGA GAC CCC AGG TAC GAA GAG TCA CTC CAC ACT CCT TAT CCT GAC AGC AGT TGG

V S G D P R Y E E S L H T P Y P D S S W

421/141 451/151

TTG AGG ACT GTG ACT ACA ACC AAA GAA TCA CTT CTC ATA ATA TCG CCC AGC ATC GTG GAA

L R T V T T T K E S L L I I S P S I V E

481/161 511/171

ATG GAT ATT TAC GGC AGG ACT CTC CAT TCC CCC ATG TTT CCT TCA GGA GTA TGT TCC AAC

M D I Y G R T L H S P M F P S G V C S N

541/181 571/191

GTA TAT CCC TCT GTC CCA TCC TGT GAG ACT AAT CAT GAT TAC ACA TTA TGG CTG CCT GAA

V Y P S V P S C E T N H D Y T L W L P E

601/201 631/211

GAT CCT AGT TTG AGT TTG GTC TGT GAT ATC TTT ACT TCC AGC AAC GGA AAG AAG GCC ATG

D P S L S L V C D I F T S S N G K K A M

661/221 691/231

AAC GGG TCA CGC ATC TGC GGA TTC AAG GAT GAA AGG GGA TTC TAC AGA TCT TTA AAG GGC

N G S R I C G F K D E R G F Y R S L K G

721/241 751/251

GCT TGC AAG CTG ACA TTG TGT GGA AGA CCT GGA ATT AGG TTA TTC GAC GGA ACT TGG GTC

A C K L T L C G R P G I R L F D G T W V

781/261 811/271 **Bcl I** ->

TCT TTT ACA AAG CCG GAC GTG CAC GTA TGG TGC ACT CCC AAC CAA TTG ATC AAT TTG CAC

S F T K P D V H V W C T P N Q L I N L H

841/281 **site III region PV** 871/291

GAC TTT CGC TCA GAC GAA ATT GAG CAC CTT GTT GTA GAG GAG TTG GTC AAG AAG AGA GAG

D F R S D E I E H L V V E E L V K K R E

901/301 931/311

GAG TGT CTG GAT GCA CTA GAG TCC ATC ATG ACC ACC AAG TCA GTG AGT TTC AGA CGT CTC

E C L D A L E S I M T T K S V S F R R L

961/321 991/331

AGT CAT TTA AGA AAA CTT GTC CCT GGG TTT GGA AAA GCA TAT ACC ATA TTC AAC AAG ACC

S H L R K L V P G F G K A Y T I F N K T

1021/341 1051/351

TTG ATG GAA GCC GAT GCT CAC TAC AAG TCA GTC AGA ACT TGG AAT GAG ATC ATC CCT TCA

L M E A D A H Y K S V R T W N E I I P S

1081/361 1111/371

AAA GGG TGT TTA AGA GTT GGG GGG AGG TGT CAT CCT CAT GTA AAC GGG GTA TTT TTC AAT

K G C L R V G G R C H P H V N G V F F N

1141/381 1171/391

GGT ATA ATA TTA GGA CCT GAC GGC AAT GTC TTA ATC CCA GAG ATG CAA TCA TCC CTC CTC

G I I L G P D G N V L I P E M Q S S L L

1201/401 1231/411

CAG CAA CAT ATG GAG TTG TTG GTA TCC TCG GTT ATC CCC CTT ATG CAC CCC CTG GCA GAC

Q Q H M E L L V S S V I P L M H P L A D

1261/421 1291/431

CCG TCT ACC GTT TTC AAG AAC GGT GAC GAG GCT GAG GAT TTT GTT GAA GTT CAC CTT CCC

P S T V F K N G D E A E D F V E V H L P

1321/441 1351/451 ->

GAT GTG CAC GAA CGG ATC TCA GGA GTT GAC TTG GGT CTC CCG AAC TGG GGG AAG TAT GTA

D V H E R I S G V D L G L P N W G K Y V

1381/461 **Transmembrane domain** 1411/471 ->

TTA CTG AGT GCA GGG GCC CTG ACT GCC TTG ATG TTG ATA ATT TTC CTG ATG ACA TGC TGG

L L S A G A L T A L M L I I F L M T C W

1441/481 **Cytoplasmic domain** 471/491

AGA AGA GTC AAT CGA TCG GAA CCT ACA CAA CAC AAT CTC AGA GGG ACA GGG AGG GAG GTG

R R V N R S E P T Q H N L R G T G R E V

1501/501 1531/511

TCA GTC ACT CCC CAA AGC GGG AAG ATC ATA TCT TCA TGG GAA TCA TAC AAG AGC GGG GGT

S V T P Q S G K I I S S W E S Y K S G G

1561/521

GAG ACC GGA CTG TGA

E T G L *

Appendix III. 3.: Open reading frame of the PVIII gene on the pPVIII plasmid.

EcoR I

1/1 **Peptide signal** 31/11 ->

ATG	GTT	CCT	CAG	GCT	CTC	CTG	TTT	GTA	CCC	CTT	CTG	GTT	TTT	CCA	TTG	TGT	TTT	GGG	AAG
M	V	P	Q	A	L	L	F	V	P	L	L	V	F	P	L	C	F	G	K

61/21 **Ectodomain: site III region** 91/31

AAT	TCC	CCC	CCC	GGT	CAG	TTG	ATC	AAT	TTG	CAC	GAC	TTT	CGC	TCA	GAC	GAA	ATT	GAG	CAC
N	S	P	P	G	Q	L	I	N	L	H	D	F	R	S	D	E	I	E	H

121/41 151/51

CTT	GTT	GTA	GAG	GAG	TTG	GTC	AAG	AAG	AGA	GAG	GAG	TGT	CTG	GAT	GCA	CTA	GAG	TCC	ATC
L	V	V	E	E	L	V	K	K	R	E	E	C	L	D	A	L	E	S	I

181/61 211/71

ATG	ACC	ACC	AAG	TCA	GTG	AGT	TTC	AGA	CGT	CTC	AGT	CAT	TTA	AGA	AAA	CTT	GTC	CCT	GGG
M	T	T	K	S	V	S	F	R	R	L	S	H	L	R	K	L	V	P	G

241/81 271/91

TTT	GGA	AAA	GCA	TAT	ACC	ATA	TTC	AAC	AAG	ACC	TTG	ATG	GAA	GCC	GAT	GCT	CAC	TAC	AAG
F	G	K	A	Y	T	I	F	N	K	T	L	M	E	A	D	A	H	Y	K

301/101 331/111

TCA	GTC	AGA	ACT	TGG	AAT	GAG	ATC	ATC	CCT	TCA	AAA	GGG	TGT	TTA	AGA	GTT	GGG	GGG	AGG
S	V	R	T	W	N	E	I	I	P	S	K	G	C	L	R	V	G	G	R

361/121 391/131

TGT	CAT	CCT	CAT	GTA	AAC	GGG	GTA	TTT	TTC	AAT	GGT	ATA	ATA	TTA	GGA	CCT	GAC	GGC	AAT
C	H	P	H	V	N	G	V	F	F	N	G	I	I	L	G	P	D	G	N

421/141 451/151

GTC	TTA	ATC	CCA	GAG	ATG	CAA	TCA	TCC	CTC	CTC	CAG	CAA	CAT	ATG	GAG	TTG	TTG	GTA	TCC
V	L	I	P	E	M	Q	S	S	L	L	Q	Q	H	M	E	L	L	V	S

481/161 511/171

TCG	GTT	ATC	CCC	CTT	ATG	CAC	CCC	CTG	GCA	GAC	CCG	TCT	ACC	GTT	TTC	AAG	AAC	GGT	GAC
S	V	I	P	L	M	H	P	L	A	D	P	S	T	V	F	K	N	G	D

541/181 571/191

GAG	GCT	GAG	GAT	TTT	GTT	GAA	GTT	CAC	CTT	CCC	GAT	GTG	CAC	GAA	CGG	ATC	TCA	GGA	GTT
E	A	E	D	F	V	E	V	H	L	P	D	V	H	E	R	I	S	G	V

601/201 -> 631/211 **Transmembrane domain**

GAC	TTG	GGT	CTC	CCG	AAC	TGG	GGG	AAG	TAT	GTA	TTA	CTG	AGT	GCA	GGG	GCC	CTG	ACT	GCC
D	L	G	L	P	N	W	G	K	Y	V	L	L	S	A	G	A	L	T	A

661/221 691 -> **Cytoplasmic domain**

TTG	ATG	TTG	ATA	ATT	TTC	CTG	ATG	ACA	TGC	TGG	AGA	AGA	GTC	AAT	CGA	TCG	GAA	CCT	ACA
L	M	L	I	I	F	L	M	T	C	W	R	R	V	N	R	S	E	P	T

721/241 751/251

CAA	CAC	AAT	CTC	AGA	GGG	ACA	GGG	AGG	GAG	GTG	TCA	GTC	ACT	CCC	CAA	AGC	GGG	AAG	ATC
Q	H	N	L	R	G	T	G	R	E	V	S	V	T	P	Q	S	G	K	I

781/261 811/271

ATA	TCT	TCA	TGG	GAA	TCA	TAC	AAG	AGC	GGG	GGT	GAG	ACC	GGA	CTG	TGA
I	S	S	W	E	S	Y	K	S	G	G	E	T	G	L	*

Appendix III. 4.: Open reading frame of the EBL1/PV gene on the pEBL1/PV plasmid.

1/1 **Signal peptide PV** 31/11 ->

ATG GTT CCT CAG GCT CTC CTG TTT GTA CCC CTT CTG GTT TTT CCA TTG TGT TTT GGG AAG
M V P Q A L L F V P L L V F P L C F G K

61/21 **Ectodomain: site II region EBL1** 91/31 **BstE II**

AAT TTC CCA ATC TAC ACC ATC CCG GAT AAA ATC GGA CCG TGG TCA CCT ATT GAT ATA AAC
N F P I Y T I P D K I G P W S P I D I N

121/41 151/51

CAT CTC AGC TGC CCA AAC AAC TTG ATC GTG GAA GAT GAG GGA TGC ACA ACT CTC ACC CCA
H L S C P N N L I V E D E G C T T L T P

181/61 211/71

TTC TCG TAC ATG GAA CTG AAA GTG GGA TAT ATA ACA ACA ATA AAA ATA GAG GGA TTT ACA
F S Y M E L K V G Y I T T I K I E G F T

241/81 271/91

TGC ACT GGT GTG ATA ACA GAG GCA GAG ACC TAC ACA AAC TTT GTA GGG TAT GTG ACT ACA
C T G V I T E A E T Y T N F V G Y V T T

301/101 331/111

ACC TTC AAG AGG AAG CAT TTC CGG CCA ACT GTG AGC GCC TGT AGA GAT GCA TAC AAC TGG
T F K R K H F R P T V S A C R D A Y N W

361/121 391/131

AAG ATT ACT GGT GAT CCG AGA TAT GAA GAG TCG CTG CAC AAT CCT TAC CCT GAC TCT CAT
K I T G D P R Y E E S L H N P Y P D S H

421/141 451/151

TGG TTG AGA ACT GTG AAG ACC ACC AAA GAG TCT CTT CTG ATT ATC TCT CCT AGT GTA GTT
W L R T V K T T K E S L L I I S P S V V

481/161 511/171

GAC ATG GAC GCA TAT GAC AAA AAT CTC TAT TCC AAA ATG TTC CCC AAT GGT AAA TGT CTG
D M D A Y D K N L Y S K M F P N G K C L

541/181 571/191

GCT TCA CCC CCA AGT GCC ACA TGT TGT CCG ACA AAT CAT GAC TAC ACC ATT TGG ATT CCA
A S P P S A T C C P T N H D Y T I W I P

601/201 631/211

GAG AAT CCT AAG CCC GGG CTG TCC TGT GAT ATA TTC ACG ACT AGC AAA GGA AAG AAA GCA
E N P K P G L S C D I F T T S K G K K A

661/221 691/231

ACC AAG GAT GGA AAG CTG TGT GGA TTT GTG GAT GAG AGA GGA TTG TAC AAA TCC TTG AAG
T K D G K L C G F V D E R G L Y K S L K

721/241 751/251

GGG GCT TGC AAG CTG AGA CTC TGT GGG GTC CCT GGC ATG AGA TTG ATG GAT GGA TCA TGG
G A C K L R L C G V P G M R L M D G S W

781/261 811/271 **EcoR I** -> **site III region PV**

GTA TCT CTT CAG AAA ACC GAA GCT CCA GAA TGG TGC TCG AAT TCC CCC CCC GGT CAG TTG
V S L Q K T E A P E W C S N S P P G Q L

841/281 871/291

ATC AAT TTG CAC GAC TTT CGC TCA GAC GAA ATT GAG CAC CTT GTT GTA GAG GAG TTG GTC
I N L H D F R S D E I E H L V V E E L V

901/301 931/311

AAG AAG AGA GAG GAG TGT CTG GAT GCA CTA GAG TCC ATC ATG ACC ACC AAG TCA GTG AGT
K K R E E C L D A L E S I M T T K S V S

961/321 991/331

TTC AGA CGT CTC AGT CAT TTA AGA AAA CTT GTC CCT GGG TTT GGA AAA GCA TAT ACC ATA
F R R L S H L R K L V P G F G K A Y T I

1021/341 1051/351

TTC AAC AAG ACC TTG ATG GAA GCC GAT GCT CAC TAC AAG TCA GTC AGA ACT TGG AAT GAG
F N K T L M E A D A H Y K S V R T W N E

1081/361 1111/371

ATC ATC CCT TCA AAA GGG TGT TTA AGA GTT GGG GGG AGG TGT CAT CCT CAT GTA AAC GGG
I I P S K G C L R V G G R C H P H V N G

1141/381 1171/391

GTA TTT TTC AAT GGT ATA ATA TTA GGA CCT GAC GGC AAT GTC TTA ATC CCA GAG ATG CAA
V F F N G I I L G P D G N V L I P E M Q

1201/401 1231/411

TCA TCC CTC CTC CAG CAA CAT ATG GAG TTG TTG GTA TCC TCG GTT ATC CCC CTT ATG CAC

S S L L Q Q H M E L L V S S V I P L M H

1261/421 1291/431

CCC CTG GCA GAC CCG TCT ACC GTT TTC AAG AAC GGT GAC GAG GCT GAG GAT TTT GTT GAA

P L A D P S T V F K N G D E A E D F V E

1321/441 1351/451

GTT CAC CTT CCC GAT GTG CAC GAA CGG ATC TCA GGA GTT GAC TTG GGT CTC CCG AAC TGG

V H L P D V H E R I S G V D L G L P N W

1381/461 -> 1411/471 **Transmembrane domain**

GGG AAG TAT GTA TTA CTG AGT GCA GGG GCC CTG ACT GCC TTG ATG TTG ATA ATT TTC CTG

G K Y V L L S A G A L T A L M L I I F L

1441/481 -> 1471/491

ATG ACA TGC TGG AGA AGA GTC AAT CGA TCG GAA CCT ACA CAA CAC AAT CTC AGA GGG ACA

M T C W R R V N R S E P T Q H N L R G T

1501/501 1531/511 **Cytoplasmic domain**

GGG AGG GAG GTG TCA GTC ACT CCC CAA AGC GGG AAG ATC ATA TCT TCA TGG GAA TCA TAC

G R E V S V T P Q S G K I I S S W E S Y

1561/521

AAG AGC GGG GGT GAG ACC GGA CTG TGA

K S G G E T G L *

APPENDIX III. 5.

Oligonucleotide inserts (5'->3')

oligo träger-up	AATTCTAGAGCCGCCACCATGGTT CCTCAGGCTCTCCTGTTTGTACCC CTTCTGGTTTTTCCATTGTGTTTTG GGAAGAATTCCCCCCCCGGTCAGT T
oligo träger-down	GATCAACTGACCGGGGGGGGAAT TCTTCCCAAAACACAATGGAAAAA CCAGAAGGGGTACAAACAGGAGA GCCTGAGGAACCATGGTGGCGGC TCTAG
oligo EBL1 A - up	AATTTCCCAATCTACACCATCCCG GATAAAATCGGACCGTGGTCACCT ATTCCG
oligo EBL1 A - down	AATTCGGAATAGGTGACCACGGTC CGATTTTATCCGGGATGGTGTAGA TTGGGA

APPENDIX III. 6.

Primer used for PCR, cloning and sequencing

primer	sequence (5'->3')	strand
pCIn 5	CTCCACAGGTGTCCACTCCC	+
EBL1II	GAGGGATGCACAACTCTCACCCC	+
träger control	GGTGGTCATGATGGACTCTA	-
GH7	AGTTTCAGACGTCTCAGTCA	+
GCH4	CCCCAGTTCGGGAGACC	-
pCIn-R	CCTCCCACATCTCCCCCTGAACC	-
primer EBL1 B up	ccgtggtcaccattgatataaaccatctCAGCTGCCCAAACAACTTGATCGTGGAAGATGAG	+
primer EBL1 B down	ggaattcgaGCACCATTCTGGAGCTTC	-
M13/pUC Sequencing primer (-40)	GTTTTCCCAGTCACGAC	+

Non-matching sequences are written in small lettres. Introduced restriction sites are underlined.

APPENDIX III. 7.

Sequencing of pPVIII (insertion of oligo at 1123-1194 of the plasmid)

primer	region sequenced	strand sequenced
pCIn 5	1102 - 1471	+

Sequencing of pEBL1/PV (ORF on the plasmid: 1141-2730)

primer	region sequenced	strand sequenced
pCIn 5	1100 - 1530	+
EBL1II	1428 - 1780	+
träger control	1600 - 2010	-
GCH4	2000 - 2444	-
GH7	2098 - 2591	+
pCInR	2468 - 2860	-

APPENDIX IV: Individual data of the results shown in chapter IV.

Table IV. 1.

Immunization	VNA against CVS (IU/ml)
Gs-Bac	3.2
G-Bac	0.4 / 0.2
G-Bac in liposomes	1.6 / 6.4
IPRV	7.2 / 38

Table IV. 2.

Immunization	IL-2 after stimulation (cpm)			Anti-RNP antibodies (U/ml)
	medium	RNP	IPRV	
RNP	1218 / 6512	72770 / 78982	30665 / 45914	36.8
N-Bac	1264 / 5663	8793 / 74158	3963 / 46287	32.8

Table IV. 3.

Immunization	Protection (x mice / y mice)
RNP + CFA	5 / 16
RNP / RNP	8 / 10
RNP + CFA / RNP	10 / 10
N-Bac + CFA	0 / 8
N-Bac / RNP	3 / 10
N-Bac + CFA / RNP	3 / 9

Figure IV. 4.

Immunization	VNA against CVS (IU/ml)
Gs-Bac	1.6 / 0.9
Gs-Bac+N-Bac	1.6 / 2.0
(Gs-Bac)US	0.7 / 0.9
(Gs-Bac+N-Bac)US	8.9 / 2.3
IPRV	21.7 / 16.6

Figure IV. 5.

Immunization	VNA against CVS (IU/ml)
G-Bac	0 / 0.7
G-Bac+ 0.01 µg N-Bac	0.2 / 0.3
G-Bac+ 0.1 µg N-Bac	0.7 / 1.0
G-Bac+ 1 µg N-Bac	1.0 / 0.2
(G-Bac)L	0.3 / 0.3
(G-Bac+ 0.01 µg N-Bac)L	0.4 / 0.6
(G-Bac+ 0.1 µg N-Bac)L	0.9 / 0.5
(G-Bac+ 1 µg N-Bac)L	0.7 / 0.2
IPRV	7.2 / 38

Figure IV. 6.

VNA against EBL1 (IU/ml)			VNA against PV (IU/ml)		
day 14	day 21	day 40	day 14	day 21	day 40
1.4	3.2	3.2	2.5	3.3	4.7
3.2	3.2	3.2	3.3	5.1	7.8
0.8	1.6	3.2	3.3	2.2	3.7
1.6	1.6	3.2	3.3	3.3	0.8
0.5	1.6	3.2	2.2	2.2	7

Figure IV. 7.

Immunization	IL-2 after stimulation (U), day 14				day 21			
	medium	EBL1	PV	ConA	medium	EBL1	PV	ConA
pEBL1/PV	0 / 0	0.16 / 0.16	0.2 / 0.58	2.4 / 3	0 / 0	0.2 / 0.33	0.62 / 0.16	4.63 / 4.69
pPV/PV	0 / 0	0.16 / 0.08	0.3 / 0.33	3.5 / 2.5	0 / 0	0 / 0	0.55 / 0.55	10 / 10
pCI-neo	0 / 0	0 / 0	0 / 0	3.5 / 2	0 / 0	0 / 0	0 / 0	4.1 / 2.8

Table IV. 8. a.

Immunization	VNA (IU/ml) day 14 against		VNA (IU/ml) day 21 against	
	EBL1	PV	EBL1	PV
pEBL1/PV	1.6 / 0.8	0.8 / 0.4	2.6 / 6.4	1.1 / 4.3
pPV/PV	0.8 / 0.8	1.8 / 0.8	0.8 / 0.8	0.4 / 3.2
pCI-neo	0.4 / 0.4	0.2 / 0.2	0.4 / 0.4	0.2 / 0.2

Table IV. 8. b.

Immunization	Protection against EBL1	Protection against PV
pEBL1/PV	3 / 4	3 / 4
pPV/PV	0 / 5	2 / 5
PBS	0 / 5	0 / 5

Figure IV. 13 (see below)

Figure IV. 14.

Immunization	Antibodies (U/ml)
pPVIII	0.07 / 0.03
pPVIII+IL-2	0.2 / 0.21
PBS	0.05 / 0.05
PBS+IL-2	0.1 / 0.07

Figure IV. 15.

Immunization	IL-2 (U) after stimulation			
	medium	IPRV	G	ConA
pPVIII	0 / 0	0.63 / 0.3	0.2 / 0.38	0.88 / 0.92
PBS	0 / 0	0 / 0	0 / 0	2.75 / 1.0

Figure IV. 13. (legend: see figure IV. 10. and 11.; without or with fixation: no / yes)

	24 h	48 h	72 h	96 h	120 h	144 h
poly G-PV no	pPV/PV 30-40% F pEBL1/PV 60% F pPVIII 0	pPV/PV 40% F pEBL1/PV 50-60% F pPVIII 5% S	pPV/PV ²100% F pEBL1/PV ²100% F pPVIII 5-10% S	pPV/PV 60% F pEBL1/PV 50-60% F, 5% S pPVIII 10% S	pPV/PV 40-50% F, 1% S pEBL1/PV 50% F, 5-10% S pPVIII 10% S	pPV/PV 50% F pEBL1/PV 50-60% F, 5% S pPVIII 10% S
poly G-PV yes	pPV/PV 30% F pEBL1/PV 50-60% F pPVIII 5-10% (+/-)	pPV/PV 30-40% F pEBL1/PV 50-60% F, 1% S pPVIII 10% S	pPV/PV 60% F, ²1% S pEBL1/PV 60-70% F, 10% S pPVIII 10-20% S	pPV/PV 30-40% F, ²1% S pEBL1/PV 50-60% F, 10% S pPVIII yes: 20% S	pPV/PV 30-40% F, 1% S pEBL1/PV 50-60% F, 20% S pPVIII 20% S	pPV/PV 30-40% F, 1% S pEBL1/PV 50-60% F, 10% S pPVIII 20% S
PVE12 no	pPV/PV 40% F pEBL1/PV 0 pPVIII 0	pPV/PV 40-50% F pEBL1/PV 0 pPVIII 0	pPV/PV 80% F pEBL1/PV 0 pPVIII 0	pPV/PV 60% F pEBL1/PV 0 pPVIII 0	pPV/PV 40% F pEBL1/PV 0 pPVIII 0	pPV/PV 50% F pEBL1/PV 50-60% F, 5% S pPVIII 20% S
PVE12 yes	pPV/PV 30 % F pEBL1/PV 0 pPVIII 0	pPV/PV 40% F pEBL1/PV 0 pPVIII 0	pPV/PV 50% F pEBL1/PV 0 pPVIII 0	pPV/PV 30% F pEBL1/PV 0 pPVIII 0	pPV/PV 30-40% F pEBL1/PV 0 pPVIII 0	pPV/PV 30% F, 1% S pEBL1/PV 50% F, 10% S pPVIII 20% S
D1 no	pPV/PV 30-40% F pEBL1/PV 50-60% F pPVIII 0	pPV/PV 40% F pEBL1/PV 50-60% F pPVIII 0	pPV/PV ²100% F pEBL1/PV 70-80% F pPVIII 0	pPV/PV 40% F pEBL1/PV 50-60% F pPVIII 0	pPV/PV 50% F pEBL1/PV 60% F pPVIII 0	pPV/PV 50% F pEBL1/PV 0 pPVIII 0
D1 yes	pPV/PV 30% F pEBL1/PV 50-60% F pPVIII 0	pPV/PV 30-40% F pEBL1/PV 40-50% F pPVIII 0	pPV/PV 60% F pEBL1/PV 60% F pPVIII 0	pPV/PV 30% F pEBL1/PV 40% F pPVIII 0	pPV/PV 40% F pEBL1/PV 50-60% F pPVIII 0	pPV/PV 30% F pEBL1/PV 0 pPVIII 0
6-B1 no	pPV/PV 0 pEBL1/PV 0 pPVIII 0	pPV/PV 0 pEBL1/PV <1% S pPVIII 1% S	pPV/PV ²1% pEBL1/PV 1% F pPVIII 1% S	pPV/PV ²1% S pEBL1/PV 5% S pPVIII 5-10% S	pPV/PV 1-2% S pEBL1/PV 5-10% S pPVIII 5% S	pPV/PV 1-2% F pEBL1/PV 20% F pPVIII 5-10%
6-B1 yes	pPV/PV 0 pEBL1/PV 0 pPVIII 0	pPV/PV yes: 0 pEBL1/PV yes: <1% S pPVIII yes: 5-10 % S	pPV/PV ²1% pEBL1/PV 5-10% S pPVIII 10% S	pPV/PV ²1% S pEBL1/PV 10% S pPVIII 5-10% S	pPV/PV 1% S pEBL1/PV 5-10% S pPVIII 10% S	pPV/PV 1% S pEBL1/PV 10-20% S pPVIII 10% S

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AND
CURRICULUM VITAE

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PRAKTIKA

Mai/Juni, Sep. 1989	Dr. med. vet. M. Maurer, Heidelberg, ***Kleintierpraxis*** (8 W.)
März 1991	Dr. D. Wadsworth, Blackpool (Großbritannien), ***Kleintierpraxis*** (3 W.)
April 1991	Landwirtschaftliche Lehranstalten Triesdorf, ***Landwirtschaft*** (10 T.)
März 1992	Dr. B. Gombeaud, Royan (Frankreich), ***Kleintierpraxis*** (4 W.)
Juli/Aug. 1993	Dr. M. Finelle, Semur-en-Auxois (Frankreich), ***Gemischtpraxis*** (6 W.)
Juli/Aug. 1994	Veterinärdienst der Stadt Mannheim, ***Schlachthof*** (6 W.)
Okt. - Dez. 1994	Dr. L.Vogelnest, Taronga Zoo, Sydney, Australien, ***Wildtiere*** (6 W.) Dr. D. Schultz, Adelaide Zoo, Adelaide, Australien, ***Wildtiere*** (6 W.)

BERUFSERFAHRUNG

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