Review Article

Rabies Subunit Vaccines

By William H. Wunner,* Bernhard Dietzschold, Peter J. Curtis and Tadeusz J. Wiktor

The Wistar Institute, 36th Street at Spruce, Philadelphia, Pennsylvania 19104, U.S.A.

Introduction

Rabies virus vaccines are administered mainly to animals for prophylaxis against rabies virus infection, to humans in post-exposure treatment, and to veterinarians and laboratory workers. Although the mechanism of protection following vaccination with inactivated rabies virus and live attenuated viruses in animals and humans remains poorly understood, it is clear that humoral and cellular immune responses are induced by virus vaccine in immunized animals. High levels of virus-neutralizing (VN) antibodies induced by vaccination before exposure to rabies virus usually assures protection against subsequent lethal virus challenge (Sikes et al., 1971) and would therefore seem to play a role. However, the protective effect in post-exposure treatment is only produced by vaccine and not by anti-rabies serum alone which indicates that vaccine activates a second protective mechanism. Inactivated rabies virus vaccine induces a strong specific cell-mediated cytotoxic (CMC) response in mice which is a function of thymus-derived virus-immune T cells (Wiktor et al., 1977; Wiktor, 1978). The fact that challenge of vaccinated animals with street rabies virus resulted in a secondary CMC response implies that stimulation of CMC is possibly the most important factor in the defence mechanism of the host, since the level of CMC response, rather than VN antibodies, is closely correlated with protection (Wiktor, 1978). Interferon induction by vaccine may also play a role in the mechanism of post-exposure protection; however, its precise function is not yet fully understood (Wiktor et al., 1972, 1976).

Subunit vaccines consisting of the immunogenic component of a specific virus or merely its immunoreactive portion were considered, for practical purposes, to be a rather futuristic perception of the ultimate in safe (i.e. genome-free) antiviral vaccines. Recently, with the progress in recombinant DNA technology and increasing facility for the chemical synthesis of short peptides of specific amino acid sequences, the concept of a subunit vaccine has generated considerable research activity directed towards identifying the essential immunogenic determinants of viruses. The biosynthesis in Escherichia coli of an immunogenic analogue of the VP1 protein of foot-and-mouth disease virus (FMDV) fused to an E. coli protein (Kleid et al., 1981) and chemical synthesis of a small immunogenic peptide whose sequence was derived from a specific region in VP1 of FMDV (Bittle et al., 1982) are prime examples of the results that might be expected. Those viruses which derive their immunogenicity from external spike projections of multimeric structures on the membrane surface, however, may present a more complex array of determinants to the immune system than do viral capsid proteins of a non-enveloped virus. We have been examining the major surface antigen of rabies virus to determine its immunogenic and antigenic properties. Our recently determined nucleotide sequence of the gene encoding the rabies virus-specific glycoprotein (G protein) from which the amino acid sequence has been deduced (Anilionis et al., 1981) provides a basis for mapping immunogenic determinants of G protein. In this review of rabies virus G protein studies, we discuss considerations that might influence the strategy for developing a subunit rabies vaccine.

Rabies virus glycoprotein as an immunogenic subunit

It has been firmly established that rabies virus G protein forms spike projections on the external surface of the virus membrane and is the major antigen responsible for the induction of VN antibodies and for conferring immunity against lethal infection with rabies virus (Crick &
Brown, 1969, 1970; Wiktor et al., 1973; Cox et al., 1977). To study the biological and antigenic nature of the rabies virus G protein, purified virus was disrupted with Triton X-100, and G protein was isolated from other viral membrane protein and lipid components in an isoelectric-focusing column (Dietzschold et al., 1974). Purified G protein focused at pI 7 and was homogeneous with respect to molecular composition and size. The mol. wt. of G protein isolated under non-denaturing conditions (in Triton X-100) was approximately 400000. Under denaturing conditions for SDS-polyacrylamide gel electrophoresis (SDS-PAGE), the same material was found to consist solely of G protein with mol. wt. 80000 (Dietzschold et al., 1978).

Further characterization of non-denatured purified G protein showed that it retained reactivity with specific antisera in a complement fixation test but, in contrast to intact virions, failed to haemagglutinate goose erythrocytes. Electron microscopic examination of G protein in the presence of Triton X-100 revealed an evenly dispersed viral protein preparation (Cox et al., 1980). When the Triton X-100 was decreased in exchange for n-octyl-β-D-glucopyranoside (n-octyl glucoside) followed by removal of the second detergent by dialysis, Cox and co-workers showed that the purified G protein aggregated to form rosette structures of variable size and shape. The structures were composed of an electron-lucent ring 5 to 10 nm thick surrounding amorphous material that occasionally had an electron-dense centre and revealed surface projections with an average length of 8 nm. The interesting observation was that these structures retained the haemagglutinating activity of the intact virion.

A particularly significant finding in their study supports the basic concept of a rabies subunit vaccine. The rosette structures of G protein (haemagglutinin preparation) which consisted solely of polypeptide chains of G protein was fully protective against a lethal challenge infection with rabies virus in mice. The pre-exposure protective and antigenic values of this haemagglutinin preparation were equal to that of inactivated virus vaccine and at least ten times greater than those for the monomeric form of G protein in Triton X-100. One further important note stems from the kinetics of protection induced by the haemagglutinin preparation compared with virus vaccine. After a single dose, the pre-exposure protection induced by the haemagglutinin preparation was delayed. Brown & Crick (1974) previously observed a delay in the antibody response in guinea-pigs following inoculation of rabies subunits. This clearly is an undesirable characteristic, especially since a rapid establishment of immunity is essential in post-exposure treatment of rabies virus infection. In contrast to these findings, preliminary tests have indicated that reconstituted vesicles containing viral lipids and G protein (virosomes) confer an early protection (Cox et al., 1980; B. Dietzschold et al., unpublished results). Since the orientation of G protein molecules in association with viral membrane lipids in virosomes is similar to that in the virus particle (Perrin & Atanasia, 1981), one might suggest that G protein is not only required, but a higher order association of G protein molecules is more effective for immunogenicity.

**Structural requirements for immunogenic activity of G protein**

The possibility that only a portion of the native viral antigen may be required to induce the protective response against lethal challenge infection is both scientifically intriguing and economically advantageous. The latter is especially important from a manufacturing point of view, particularly with regard to producing subunit antiviral vaccines biosynthetically or by methods that involve a chemical synthesis process. Other important advantages in manufacture of subunit vaccine are safety, the easier potency testing of a chemically defined product, and ease of storage and transportation especially in the Third World. In order to determine the structural basis of the immunogenic activity of the native G protein and perhaps define the important regions of the active subunit molecule, we have examined the structure and tested the immunological activity of both naturally occurring and chemically cleaved fragments of ERA strain rabies virus G protein. Rabies virus-infected cells shed a soluble glycoprotein (Gs protein) which has been purified from virion-depleted tissue culture fluid by immunoadsorption chromatography (Dietzschold et al., 1983). We have compared Gs protein with the virion-associated G protein which has been solubilized in 2% Triton X-100 and similarly purified by immunoadsorption chromatography. In SDS-PAGE the apparent mol. wt. of the faster
migrating Gs protein was 61 000 as compared to 67 000 for G protein. When the two proteins were tested for their ability to bind monoclonal VN antibodies that recognized four distinct antigenic sites of ERA strain G protein, no antigenic difference was detected between Gs and G proteins. We also investigated whether the antigenically similar Gs protein would confer the same protection as G protein against lethal challenge infection with rabies virus in immunized mice and found that Gs protein failed to protect mice even at the highest antigen concentration used (Dietzschold et al., 1983). The VN antibody titre at the time of challenge following immunization with Gs protein was 15 times lower than the VN antibody titre developed by immunization with G protein.

The difference in the molecular weights of Gs and G proteins may be the important factor accounting for their disparate immunogenicities. To analyse the structural relationship between Gs and G proteins, [3H]leucine-labelled Gs protein and [14C]leucine-labelled G protein were mixed and exhaustively digested with trypsin (Dietzschold et al., 1983). The leucine-containing tryptic peptides were separated by high pressure liquid chromatography on a reverse phase column, and the profiles indicated that two tryptic peptides of G protein were missing from Gs protein. By determining the sequence of the first nine amino acids from the amino-terminus of one of the tryptic peptides obtained from G protein and absent from Gs tryptic peptides, the peptide was mapped to amino acid residues numbered 467 to 475 in the deduced amino acid sequence of G protein (Anilionis et al., 1981). This finding was immediately significant, since it indicated that the peptide missing in Gs protein corresponded to a portion of the cytoplasmic domain of G protein, and could explain how the antigenic sites which bound monoclonal VN antibodies similarly on the two proteins were unaffected if all sites on the molecules were located on the external (amino-terminal) side of the transmembrane segment. The portion of G protein which was missing from Gs protein was precisely determined by enzymically sequencing the carboxy-terminal amino acids of Gs protein. The sequence obtained matched residues numbered 444 to 447 in the predicted sequence, located within the first eight amino acids of the putative transmembrane segment shown in Fig. 1. This indicated that 58 amino acid residues from the carboxy-terminus of G protein were missing in Gs protein. Automated amino-terminal sequencing of the first 10 amino acid residues revealed that the amino-terminal region of Gs protein was identical to that of G protein (Dietzschold et al., 1983).

The 58 amino acids missing in Gs protein accounted for a 6200 dalton difference between Gs protein and G protein. Since a 6200 dalton fragment was all that was required to account for the size difference between Gs and G proteins, no difference in glycosylation was expected between the two antigens. A similar comparison exists between the Gs and G proteins of vesicular stomatitis virus (Little & Huang, 1978; Irving & Ghosh, 1982). The low immunogenicity of Gs protein is probably structurally related to that portion of the molecule which is missing compared with G protein. Whereas G protein aggregated at low concentrations of n-octyl glucoside and was immunogenic in mice, Gs protein did not aggregate and was poorly immunogenic in mice. This was a significant finding and one which has several implications for vaccine development. Firstly, in practical terms, evaluations of virus vaccine potency which are based solely on tests that quantify viral antigens, such as by measuring the binding of anti-G protein antibodies (Atanasiu et al., 1980) or by quantification in a radial-immunodiffusion test (Ferguson & Schild, 1982), will result in an overestimation of potency when Gs protein is present in the virus vaccine preparation unless the presence of Gs is determined separately (Atanasiu et al., 1982). Secondly, a portion of the hydrophobic transmembrane segment or carboxy-terminal fragment in G protein which is missing in Gs may exert an adjuvant effect. Thirdly, the architectural design of a rabies subunit vaccine derived from synthetic or recombinant DNA technologies will have to take into consideration the difference in immunogenicity between G and Gs proteins.

To study the immunogenicity of smaller peptide fragments of known amino acid sequence, the rabies virus G protein has been chemically cleaved with cyanogen bromide (CNBr) into a limited number of large CNBr peptides. These were fractionated into seven peptide bands under reducing conditions (Cr1 to Cr7) or six peptide bands under non-reducing (carboxymethylated) conditions (Ca1 to Ca6) (Dietzschold et al., 1982). The reduced peptides were separated by SDS--
Fig. 1. Comparison of G protein and G protein of the ERA strain of rabies virus and a map of CNBr peptides of G protein. The entire amino acid sequence of G protein, including the signal peptide (SP), was deduced from the nucleotide sequence of a cDNA copy of the G protein mRNA sequence. The amino acids are in single letter code. The four enzymically determined carboxy-terminal amino acids of G protein are shown in white letters on a black background located within the transmembrane (TM) region. The order of eight reduced CNBr peptides of G protein within the deduced amino acid sequence is shown by bracketed bold letters. The deduced sequences of the unrecovered CNBr peptides and cytoplasmic fragment are indicated by light letters. Three glycosylation signals, N-(X)-S and N-(X)-T, on the amino-terminal side of TM are underlined; the putative glycosylation sites are indicated by arrows.

PAGE and recovered from the gel to determine the amino-terminal sequence of each peptide. By matching the first 7 to 12 amino acids with the predicted amino acid sequence for the G protein, the individual CNBr peptides have been mapped within the G protein sequence (see Fig. 1). After each peptide band from the gel had been subjected to automated Edman degradation, only one peptide band (designated Cr2 in the resolving gel) produced two distinct residues at each degradation step, indicating that this band consisted of two peptides, Cr2 and Cr2-A. Thus, eight large CNBr peptide fragments ranging in size from 32 to 77 amino acids in length were mapped within the G protein sequence. Five smaller CNBr peptides containing 6, 7, 8, 9 and 12 amino acid residues and the methionine-free carboxy-terminal fragment...
containing 47 residues of the G protein were not recovered. Two of the large CNBr peptides (Cr3 and Cr4) were immunoprecipitated with rabbit hyperimmune anti-rabies serum. To determine whether the purified CNBr peptide fragments were immunogenic, individual reduced peptides were injected into mice, and the induced antibodies were evaluated. We found that all of the peptides induced significant titres of antibodies that bound in radioimmunoassay to virus and to purified viral G protein (both native and denatured). In addition, antibodies induced by Cr1, Cr3 and Cr4 exhibited VN activity with titres of 180, 270 and 510 respectively, and were active in complement-dependent lysis with comparable titres. None of the peptides was coupled to high molecular weight carriers which might account for the low VN activity of these CNBr peptide-induced antibodies compared to native G protein. Another explanation for the relatively low VN antibody titres may be that the conformational structure of G protein, which contributes greatly to its antigenic and immunogenic activity, was presumably lost during isolation of the peptides. This line of reasoning is supported by our observations that monoclonal VN antibodies did not bind to the reduced CNBr peptide fragments or that after mild reduction, the native G protein lost 95% of its antigenic activity (B. Dietzschold et al., unpublished results).

To relate the decreased antigenicity to a reduction of intramolecular disulphide bridges in native G protein, CNBr peptides carboxymethylated with iodoacetamide and isolated under non-reducing conditions were subsequently reduced and analysed for peptide–peptide interactions (Dietzschold et al., 1982). It was found that two electrophoretically separated bands of alkylated CNBr peptides (Ca1 and Ca2) were each composed of two CNBr peptides according to electrophoretic analysis under reducing conditions and N-terminal amino acid sequencing data. Two interesting possible configurations are manifested by the two di-peptide interactions. The results indicated that all of one reduced CNBr peptide fragment (Cr6) was linked by disulphide bonds either to Cr1 or Cr2 in the non-reduced form. At the same time, some of the Cr1 and Cr2 peptides remained unlinked to Cr6. These findings suggest that monomers of rabies G protein exist in two different conformations. A simple model of the rabies G protein spike that depicts the two conformations simultaneously would be a multimeric structure in which peptide Cr6 is linked to Cr1 forming a large loop in at least one monomer and peptide Cr6 is linked to Cr2 forming a smaller loop in one or more other monomers. It is presumed that these two conformations would have significance for the assembly of the G protein spike and also provide a complex array of determinants to the immune system of the virus-infected host.

The level of VN antibody titres raised against G protein was 10 to 100 times higher than those produced by CNBr peptide fragments and Gs protein. Precisely how the immunodominant site(s) on rabies virus G protein is affected by the gradual unfolding (denaturation) and cleavage is not entirely clear from these experiments. We may assume that the native support structure that contributed to the immunizing activity was progressively altered and that a systematic process of reconstructing the immunogenic component from subunit fragments of G protein will have to be developed in order to understand the significance of these changes. There are perhaps two levels of reconstruction. Firstly, for a subunit peptide vaccine to be as effective as the native spike G protein, it must be made to fold properly even when deprived of its native support structure. Secondly, it would appear that a properly folded peptide might also have to be aggregated into a suitably large particle in order that it might acquire its full immunogenic activity. This might be achieved by coupling the peptide with a hydrophobic ‘tail’ (protein or lipid) that provides an adjuvant effect, presumably by formation of aggregates, which serves to enhance the immune response in immunized animals. This is supported by the observation that dissociated G protein did not induce T cell-mediated cytotoxicity (Wiktor, 1978). Induction did occur when G protein was inserted into lipid vesicles (R. I. Macfarlan et al., unpublished results).

A rabies subunit vaccine by the synthetic or recombinant DNA approach

The question under consideration is whether or not it will be possible to achieve protection against rabies virus infection by immunization with a chemically synthesized or recombinant DNA-expressed polypeptide. The results obtained with a chemically synthesized peptide corresponding to a specific region of the VP1 polypeptide of FMDV (Bittle et al., 1982) suggest
that under certain circumstances a single peptide can elicit a response in animals sufficient to
protect them against infection with the parental virus. Although the reason for this response is
not immediately obvious, one may speculate that the antibody induced by the synthetic peptide
is more closely related qualitatively to that induced by virions than to purified VP1.

To define the antigenic and immunogenic regions of the rabies virus G protein, we cloned a
double-stranded complementary DNA (cDNA) copy of its mRNA into plasmid pBR322 (Curtis
et al., 1981). The cDNA copy was inserted into pBR322 at the PstI site by dG–dC tailing and the
complete nucleotide sequence of the G protein cDNA containing 1650 base pairs determined
(Anilionis et al., 1981). The nucleotide sequence allowed us to predict several features of the G
protein from the deduced amino acid sequence shown in Fig. 1. An open reading frame,
beginning with an initiation codon (ATG) and ending with a stop codon (TGA), suggested that
the nucleotide sequence coded for a polypeptide of 524 amino acids. However, by direct amino
acid sequence analysis of purified rabies virus G protein, the first six amino acid residues of G
protein were located in the deduced sequence at residues 20 to 25 from the putative initiating
methionine (Lai & Dietzschold, 1981). This indicated that the first 19 amino acid residues that
precede the amino-terminal lysine of the purified G protein, and which are predominantly
hydrophobic, presumably represent a signal peptide. An uninterrupted hydrophobic sequence
of 22 amino acids, bound by residues 439 (numbered from the amino-terminal lysine) and 462 is
the proposed transmembrane region. A cytoplasmic sequence which extends from the
presumptive transmembrane domain to the carboxy-terminal leucine contains 44 amino acids
that are both charged and uncharged residues. Located on the amino-terminal side of the
transmembrane domain are three carbohydrate acceptor sequences, N-(X)-S and N-(X)-T (in
single letter code).

The cloned G protein cDNA and the amino acid sequence deduced from its nucleotide
sequence provide the basis for constructing immunogenic polypeptides. Yelverton et al. (1983)
have prepared plasmids for the expression of full-length rabies G protein in E. coli. They report
expression of rabies G protein to about 2 to 5% of the cellular protein depending on whether the
G protein is unfused or part of a fused protein. However, their usefulness as immunogenic
agents was not reported. In preliminary studies of our own, protein produced by recombinant
DNA technology in E. coli (R. Lathe, unpublished results) so far has not elicited responses in
animals that could protect them against infection with rabies virus. However, expression of the
G protein gene was too inefficient to test adequately its antigenic and immunogenic properties.
Lack of glycosylation in E. coli also may be a reason for the absence of immunogenic activity
with the rabies G protein gene product. Glycosylation differences among antigenic variants of
fixed rabies virus suggest that glycosylation may contribute to antigenic structure and indirectly
affect binding of VN antibodies (W. H. Wunner et al., unpublished results).

In order to construct immunogenic determinants of rabies G protein by the chemically
synthetic peptide route, several considerations will have to be taken into account. Firstly, the
correct sequences representing the immunogenic determinant will have to be identified.
Secondly, the peptide containing the specific sequence of an immunogenic determinant must
assume the correct conformation in order to elicit VN antibody. Thirdly, a combination of two
or more determinants which might interact to provide a multi-site structure that stimulates T
and B cells of the animal’s immune system may be necessary; for example, the specific regions of
the molecule which stimulate T cells may be different from the sites which bind VN antibodies.
Such a determinant recognized by a T-helper cell line and not by VN antibodies has been
localized in the HA1 molecule of the PR8 strain of influenza virus (Hackett et al., 1983).

CONCLUSIONS

The secondary and tertiary structures of the rabies virus spike G protein are important for its
ability to induce VN antibodies and confer immunity to the host. For a subunit peptide vaccine
to be as effective as the native spike G protein, it would appear that the amino acid sequence
comprising the antigenic determinant for VN antibody binding must be made to fold properly
even when deprived of its native support structure. Since CNBr peptides have retained at least
some of their antigenicity for binding antibodies from hyperimmune serum but not monoclonal
VN antibodies, and their immunogenicity, then synthetic peptides containing corresponding sequences should show similar activities. Additionally, determinants that might be necessary for stimulating T lymphocytes would have to be built into the synthetic peptide preparation. It would also appear that a properly folded peptide might have to be aggregated into suitably large particles for it to achieve its full protective effect. Adjuvants may serve in this capacity to enhance the immune response to relevant peptides and thus improve the immunogenicity of a subunit vaccine that ultimately protects animals and humans against rabies virus infection.

This work has been supported by Research grants AI-09706, AI-18562 and AI-18883 from the National Institute of Allergy and Infectious Diseases.

REFERENCES


