Structural and Immunological Characterization of a Linear Virus-Neutralizing Epitope of the Rabies Virus Glycoprotein and Its Possible Use in a Synthetic Vaccine

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We have mapped a linear epitope recognized by the virus-neutralizing monoclonal antibody 6-15C4 within the primary sequence of the G protein from the Evelyn-Rokitnicki-Abelseth strain of rabies virus. This was accomplished by using fragments of the rabies virus G protein and deduced amino acid sequences of neutralization-resistant variant rabies viruses. The monoclonal antibody 6-15C4 specifically recognized a synthetic peptide (peptide G5-24) which resembles the 6-15C4 epitope in structure. In addition, a tandem peptide constructed from the G5-24 peptide and a dominant T_H cell epitope of the rabies virus N protein induced protective immunity against lethal rabies virus challenge infection in mice.

The glycoprotein (G) of rabies virus has been identified as a major antigen that induces protective immunity against rabies virus (5). Although protection against rabies virus infection is probably the result of many host-effector interactions (8, 22), virus-neutralizing antibodies (VNA), which are induced solely by the G protein, are believed to play a major role in immunoprotection against rabies (20). Thus, the rabies virus G protein represents a logical choice for the development of a subunit vaccine that can be used for immunization against rabies in humans and animals (6). It has already been shown that a vaccinia virus recombinant containing the rabies virus G protein gene not only induces high levels of VNA but also confers excellent protection from a lethal challenge with rabies virus (25).

The use of synthetic peptides representing selected amino acid sequences from the rabies virus G protein offers another approach for development of an efficacious vaccine. A prerequisite for this second approach is a precise knowledge of the structure of virus-neutralizing epitopes of the rabies virus G protein. By using neutralization-resistant variant viruses selected in the presence of excess amounts of neutralizing monoclonal antibodies (MAbs) and competition binding assays with G protein-specific MAbs, three distinct antigenic sites of the G protein of the challenge virus standard (CVS)-11 strain and five antigenic sites of the G protein of the Evelyn-Rokitnicki-Abelseth (ERA) strain of rabies virus could be operationally delineated (14, 15). For the tentative identification of epitopes in the rabies virus G protein, amino acid substitutions were identified in several of these mutants that correlated with lack of neutralization (17, 19, 27, 28). This analysis revealed that most of the antigenic structure of site III comprised a more continuous distribution of epitopes, since the different epitopes are closely arranged in the primary structure and even share amino acids. The structure of site II appears to be discontinuous because not all amino acid substitutions at site II in variant

viruses form a cluster within the primary sequence of the G protein (17). Antigenic site I is an independent site containing a single epitope (14, 15) not shared with epitopes of sites II, III, IV, and V. However, all virus-neutralizing MAbs known to recognize sites I to III of CVS or I to V of ERA virus do not bind at all or bind only very weakly to denatured G protein, suggesting that the conformation derived from higher-order (secondary and tertiary) structure of the G protein is required for recognition by these MAbs (26). Clearly, a precise topographical map of the conformational epitopes in site I to III of CVS virus and sites I to V of ERA virus requires knowledge of the three-dimensional structure of the G protein, and this has not yet been described.

By using two recently described MAbs (6-15C4 and 2-22C5) derived from a mouse immunized with the Pitman-Moore strain of rabies virus, a unique antigenic site on the rabies virus G protein has been identified (3). In contrast to all epitopes of rabies virus G protein described thus far, the 6-15C4 and 2-22C5 epitopes do not depend on the native conformation of the G protein for binding VNA (3). This suggests that the structure of these epitopes is indeed continuous. In this report, we identify the location of amino acids associated with these epitopes and describe the use of protein fragments as well as the deduced amino acid sequence of the G protein of neutralization-resistant variant viruses selected in the presence of MAbs 6-15C4 and 2-22C5 to map an antigenic domain within the primary sequence of the ERA virus G protein. We also show that a synthetic peptide resembling the structure of the 6-15C4 epitope is capable of binding specifically to MAb 6-15C4 in vitro and of inducing G protein-specific VNA in vivo. A synthetic peptide containing the amino acid sequences of the 6-15C4 epitope and the dominant 31D T-cell epitope (11) in tandem induced a significant protective response in C3H mice against a lethal rabies virus challenge. These data suggest that it may be possible to produce a synthetic peptide vaccine against rabies.

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MATERIALS AND METHODS

Preparation of virus and virus G protein. The neutralization-resistant variant viruses RV6-15C4 and RV2-22C5 were selected from the CVS-11 strain (Wistar stock virus) as described previously (3). The parent and the neutralization-resistant variant viruses were propagated on baby hamster kidney 21 cell monolayers and purified as described before (23). Rabies virus G protein was purified as described previously (7).

Preparation of G protein fragments. The generation of G protein fragments by cyanogen bromide (CNBr) cleavage or protease V8 digestion was recently described (9). The fragments were separated by tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described before (9). A 16% T-6% C (T, total acrylamide; C, cross-linking bisacrylamide) gel was used as separating gel, overlaid by a 2-cm 10% T-3% C gel and a 2-cm 4% T-3% C stacking gel (13). Molecular weight markers (MW-SDS-17 kit; Sigma Chemical Co., St. Louis, Mo.) were coelectrophoresed with the G protein fragments.

Electrotransfer and immunostaining of G protein fragments. Electroblotting was carried out essentially as described by Towbin et al. (21). The fragments were electrophoretically transferred to nitrocellulose paper (0.2 μ m; Schleicher & Schuell) for 2 h at 90 V and 5°C. The electrotransfer buffer was 25 mM Tris hydrochloride (Tris-HCl, pH 8.3)-1 mM dithiothreitol. After electrotransfer, the nitrocellulose membranes were rinsed with water, then immersed for 1 min in a 0.1% solution of amido black in methanol-acetic acid-water (45:10:45), and briefly destained with methanol-acetic acid-water (45:10:45). After electrotransfer, nitrocellulose membranes were washed three times with 50 mM Tris-HCl (pH 7.5)-0.15 M NaCl and then blocked with 2% normal horse serum in TPBS (0.01 M phosphate buffer [pH 7.5], containing 0.15 M NaCl and 0.05% Tween-20) for 30 min at room temperature. The blocked membranes were then washed three times with TPBS and incubated for 1 h with MAb diluted in TPBS. Following incubation with MAb, the membranes were blocked again with 2% normal horse serum in TPBS for 30 min and then incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) complex (Organon Teknica-Cappel, Malvern, Pa.). Membranes were then washed three times with 50 mM Tris-HCl, pH 6.8, and incubated in freshly prepared substrate solution (10 ml of a 0.3% solution of 4-chloro-1-naphthol [Sigma] in methanol, 40 ml of Tris-HCl [pH 6.8], and 50 μ l of 30% H₂O₂). Color development was allowed to proceed for up to 30 min until all bands were visible, and the reaction was stopped by washing with water.

Peptide sequence analysis. Peptides were eluted from nitrocellulose membrane as described previously (9). Dried peptides were dissolved in 88% formic acid and applied to a glass filter coated with Polybrene. Automated Edman degradation of the peptides was performed on an Applied Biosystems 470 A gas-phase sequencer. Phenylthiohydantoin amino acids obtained from the sequencer were dried in vacuo, dissolved in 50 μ l of acetonitrile-water (1:1), and analyzed on a Zorbax phenylthiohydantoin column as described before (12).

Peptide synthesis. Peptides were synthesized on *p*-alkoxybenzylalcohol resin, with a sixfold molar excess of Fmoc-amino acid symmetrical anhydrides (1). A BioSearch SAM 2 automated peptide synthesizer was used for chain assembly except for His, Asn, and Gln. These amino acid residues were incorporated manually through their pentafluorophenyl

esters. Amino acids and resins were purchased from Bachem, Philadelphia, Pa. Peptides were cleaved off the resin with trifluoroacetic acid-trimethanesulfonic acid-thioanisole (82:8:10, by volume), followed by precipitation with ether and dialysis in 1,000-molecular-weight-cutoff tubing (Spectrum, Los Angeles, Calif.). Results of amino acid analysis of peptides were consistent with the expected structures. Prediction of the secondary structure of peptides was done with the algorithm of Chou and Fasman (4).

Nucleotide sequence analysis of the glycoprotein genes of the variant viruses RV6-15C4 and RV2-22C5. Genome RNA was extracted from gradient-purified virus as described before (27). Synthetic deoxyribonucleotide primers were used to prime the synthesis of complementary DNA transcripts of the genome RNA in the presence of dideoxyribonucleoside triphosphates as described previously (27). DNA primers were synthesized on the Cyclone DNA synthesizer (Milligan/Biosearch, Burlington, Mass.) and purified by reverse-phase high-pressure liquid chromatography on a Beckman ODS Ultrasphere C-18 column as described before (27).

Immunization of mice with synthetic peptides. Groups of six-week-old male C3H mice (Jackson Laboratories, Bar Harbor, Maine) were injected intraperitoneally with 25 μ g of peptide in 50% complete Freund adjuvant. Fourteen days later, the mice received a booster immunization of 25 μ g of peptide in 50% incomplete Freund adjuvant. Ten days after the booster immunization, blood was collected from all immunized animals, and 3 weeks after the primary immunization, the mice were infected intramuscularly with 5×10^6 MICLD₅₀ of CVS-24 virus. Animals were observed for 3 weeks, and deaths were recorded daily.

Determination of virus antibody. The mouse antipeptide sera were heat inactivated at 56°C for 30 min. The neutralizing activity of the sera was determined as described previously (24). The reactivity of antipeptide sera with rabies G protein or ribonucleoprotein (RNP) was tested in an immunoblot assay as described before (9).

T-helper cell assay. Lymphocytes derived from lymph nodes or spleens of immune or naive mice were cultured in 150 μ l of Dulbecco modified Eagle medium supplemented with 2% fetal bovine serum and β -propiolactone (BPL)-inactivated ERA virus (ERA BPL) as described before (11).

RESULTS

G protein nucleotide and deduced amino acid sequence comparisons between the neutralization-resistant variants CVS-11 RV2-22C5 and CVS-11 RV6-15C4. Previously, MAbs 6-15C4 and 2-22C5 were used to select neutralization-resistant variants of the CVS-11 strain of rabies virus, designated RV6-15C4 and RV2-22C5, respectively, which enabled us to identify two unique antigenic determinants on the rabies virus G protein (3). To locate more precisely the epitopes recognized by MAbs 2-22C5 and 6-15C4, we determined the complete nucleotide sequence of the virion G protein gene of these two escape variants by cDNA primer extension analysis. A comparison of the amino acid sequences deduced from the nucleotide sequences revealed that each differed by a single amino acid from the parent CVS-11 virus G protein. The G protein of variant RV6-15C4 had a histidine at position 264 instead of the arginine in the parent, and variant RV2-22C5 substituted isoleucine for methionine at position 44 of the parent virus G protein.

Reactivity of MAbs 6-15C4 and 2-22C5 with G protein fragments. To analyze the fine structure of the epitopes within G protein recognized by MAbs 6-15C4 and 2-22C5,

FIG. 1. Analysis of fragments produced by enzymatic digestion of G protein with protease V8. G protein was digested with protease as described in the text, and the fragments were resolved on a 16% sodium dodecyl sulfate-polyacrylamide gel and electrotransferred to nitrocellulose. Lane a: Fragments were visualized by staining with amido black. Lanes b to d: Enzyme immunostaining of electrotransferred fragments with MABs specific for G (b, MAB 6-15C4; c, MAB 2-22C5; d, normal ascites fluid). The positions of molecular weight marker peptides are indicated ($10^3 M_r$).

the G protein was cleaved enzymatically at glutamic acid residues or chemically at methionine residues. Following enzymatic digestion of the G protein with protease V8, the fragments were separated on a 16% polyacrylamide gel. After electrotransfer to nitrocellulose paper, 12 bands were visualized by staining with amido black (Fig. 1, lane a). As demonstrated in Fig. 1, both MABs (6-15C4 and 2-22C5) recognized a fragment with an apparent molecular weight of 15,800 which was designated V1 (Fig. 1, lanes b and c). After chemical cleavage of the G protein with CNBr, the fragments were resolved on a 16% polyacrylamide gel, and after electrotransfer to nitrocellulose paper, five bands could be visualized by staining with amido black (Fig. 2, lane a). Only MAB 6-15C4 reacted with a fragment previously designated C_{R2} (8), which has an apparent molecular weight of 6,800 (Fig. 2, lane b). The NH_2 -terminal amino acid sequences of fragments V1 and C_{R2} were determined in order to map these fragments within the deduced amino acid sequence of the G protein. The NH_2 -terminal residues of fragment V1, (131)SLVIISPSSV(140) (10 residues determined), were found to match residues 131 to 140 of the deduced sequence, and the NH_2 -terminal amino acid sequence of the immunoreactive CNBr fragment C_{R2} , (244)QTS—ETK(250), corresponded to residues 244 to 250 of the deduced G protein sequence (the central residue could not be identified in this degradation step). The estimated molecular weight of peptide fragment V1 suggests that it consists of approximately 150 amino acid residues. Therefore, it is possible that the amino acid sequence at the COOH-terminal end of peptide V1 overlaps the NH_2 -terminal amino acid sequence of peptide C_{R2} .

The physical mapping of fragments V1 and C_{R2} is illustrated in Fig. 3. It is significant that residue 264 was located within the amino acid sequence of the immunoreactive fragments V1 and C_{R2} . In contrast, residue 44 was located

FIG. 2. Analysis of CNBr fragments of G protein. G protein was cleaved with CNBr, and the resulting CNBr fragments were separated on a 16% sodium dodecyl sulfate-polyacrylamide gel and electrotransferred to nitrocellulose membranes. Lane a: CNBr fragments were visualized by staining with amido black. Lanes b to d: Electrotransferred fragments were subjected to the enzyme immunostaining procedure with MABs specific for G (b, MAB 6-15C4; c, MAB 2-22C5; d, normal ascites fluid). The positions of molecular weight marker peptides are indicated ($10^3 M_r$).

far outside of these fragments. Therefore, it is likely that the arginine at position 264 is a part of the epitope recognized by MAB 6-15C4. The amino acid residue at position 44 did not appear to be directly involved in the recognition process of 2-22C5, and it is not clear from these results what is the site of MAB 2-22C5 recognition.

Antigenic and immunogenic activities of a synthetic peptide including residues 253 to 275 of the G protein. To further analyze the structure of the antigenic region of the G protein recognized by MAB 6-15C4, we synthesized a series of overlapping peptides ranging in length from 10 to 23 amino acids and corresponding to amino acid residues 253 to 275. These peptides were tested in an immunoblot assay for their binding activity with MAB 6-15C5. MAB 6-15C5 reacted strongly with peptide G5-24 (residues 253 to 275) and to a lesser extent with peptide G5-19 (residues 257 to 275) (data not shown). An immunoblot assay with rabbit antisera against peptide G5-24 conjugated to keyhole limpet hemocyanin revealed specific reactivity with rabies virus G protein (data not shown), indicating that this synthetic peptide has immunogenic properties. Therefore, peptide G5-24 was used to construct a peptide vaccine. To potentiate the immune response, the G5-24 peptide was synthesized and linearly extended with the N protein-derived peptide 31D (residues 404 to 418), which was previously shown to carry a T_H cell determinant that is immunodominant in C3H mice (11). The immunogenicity of peptides 31D, G5-24, and the tandem-constructed (colinear) peptide G5-24-31D, for which the amino acid sequences are shown in Table 1, was evaluated after injection into C3H mice. It can be deduced from Table 2 that in addition to ERA BPL-immunized mice, only mice immunized with peptides 31D and G5-24-31D produced T-helper cells that recognized rabies virus in vitro. Peptide G5-24 produced T cells that recognized only peptide G5-24 and G5-24-31D but not rabies virus in vitro.

Antiserum raised against peptide G5-24 in C3H mice exhibited low binding activity to G protein and very low

TABLE 1. Amino acid sequences of synthetic peptides which comprise the structure of antigenic determinants of the N or G protein of rabies virus

Peptide	Source protein	Amino acid sequence ^a
31D	N	(404) AVYTRIMMNGGRLKR (418) —COOH
G5-24	G	(253) PPDQLVNLHDFRSDEIEHLVVVEE (275) —COOH
G5-24-31D	G + N	PPDQLVNLHDFRSDEIEHLVVVEEAVYTRIMMNGGRLKR—COOH

^a Numbers indicate the positions of the first and last residues within the sequence.

A. Mapping of immunoreactive fragments V1 and C_R2 of the G protein

B. Comparison of the deduced amino acid sequences of the G protein of RV 6-15C4 and RV 2-22C5

peptide G5-24

FIG. 3. Delineation of epitope 6-15C4. G protein fragments recognized by MAb 6-15C4 were identified by their NH₂-terminal sequence and ordered within the deduced amino acid sequence of the G protein. The deduced amino acid sequences of escape variants RV6-15C4 and RV2-22C5 are compared, and the amino acid differences at positions 44 (RV2-22C5) and 264 (RV6-15C4) are indicated.

neutralizing activity. Immunization with peptide 31D did not result in an antibody response to rabies virus G protein or RNP, while mice immunized with peptide G5-24-31D exhibited notable virus-neutralizing activity and good binding activity not only to G protein but, unexpectedly, also to RNP (Table 2). To evaluate the protective activity, sham-immunized mice and peptide-immunized mice were challenged i.m. with rabies virus. Seventy-five percent of the mice immunized with peptide G5-24-31D survived, while all sham-immunized mice and mice immunized with peptide 31D or peptide G5-24 succumbed to rabies (Table 3).

DISCUSSION

In order to construct a synthetic vaccine against rabies virus protein, it is necessary to identify a linear epitope of the G protein that is recognized by a virus-neutralizing MAb.

In a previous communication, we have shown that two epitopes of the rabies virus G protein do not depend on a

conformation of the virus G protein that is easily denatured. These epitopes bind the 6-15C4 and 2-22C5 MAbs in a manner which suggests that they are linear (3). Here we have used fragments of the G protein, isolated following chemical and enzymatic cleavage, to localize the 6-15C4 epitope to a region within the amino acid sequence of the G protein. NH₂-terminal sequence analysis along with molecular weight determinations allowed us to map two MAb 6-15C4-positive fragments, V1 and C_R2. The physical mapping of these immunoreactive fragments indicated that the NH₂-terminal region of fragment C_R2 overlaps the COOH-terminal region of fragment V1, suggesting that the 6-15C4 epitope is located within these overlapping sequences, approximately between residues 244 and 281.

A comparison of the deduced amino acid sequences of the G proteins of the neutralization-resistant escape variants RV6-15C4 and RV2-22C5 revealed only one amino acid difference in each of the G proteins: one at position 44, and

TABLE 2. Immune response to synthetic peptides

Antigen in vivo	T-helper cell response ^a ([³ H]thymidine incorporation [cpm] ± SD)		Stimulation index	Antibody response ^b (antigen-binding titer)		
	No antigen	ERA BPL		G	RNP	VNA
ERA BPL	1,701 ± 212	4,828 ± 954	2.8	1:1,600	>1:800	1:3,600
31D	84 ± 16	202 ± 31	2.4	<1:25	<1:25	<1:5
G5-24	110 ± 13	128 ± 10	1.2	1:100	<1:25	1:9
G5-24-31D	107 ± 20	233 ± 31	2.2	1:400	1:400	1:90
CFA	100 ± 25	78 ± 12	0.8	<1:25	<1:25	<1:5

^a Groups of three mice were injected subcutaneously with 1 µg of ERA BPL or 5 µg of peptide in complete Freund adjuvant (CFA). After 7 days, draining lymph node lymphocytes were harvested, and 2 × 10⁵ lymphocytes were restimulated with 1 µg of virus per ml. After 24 h, supernatants were harvested and tested on HT-2 cells.

^b Groups of five mice were immunized intraperitoneally with 2 µg of ERA BPL or 25 µg of peptide in complete Freund adjuvant. After 3 weeks the mice were booster immunized with 2 µg of ERA BPL or 25 µg of peptide in incomplete Freund adjuvant. Two weeks after booster immunization, mice were bled, and the sera of immunization groups were pooled and tested for their binding activity with G protein or RNP in an immunoblot assay. VNA titers were determined by the rapid fluorescent focus inhibition test as described before (24).

TABLE 3. Protection against lethal challenge with rabies virus after immunization with synthetic peptides^a

Antigen	Mortality (no. dead/no. tested)
G5-24 + CFA	10/10
31D + CFA	10/10
G5-24-31D + CFA	2/8
CFA	8/8

^a Six-week-old C3H mice were challenged intramuscularly 30 days after the first immunization with 5×10^6 MICLD₅₀ of CVS-24. Animals were observed for 3 weeks, and deaths were recorded daily. CFA, Complete Freund adjuvant.

the other at position 264. The variant virus RV6-15C4, which is not neutralized by MAb 6-15C4, has a histidine at position 264 instead of the arginine which is found in the variant RV2-22C5 and parent G proteins. Since arginine 264 is located within the overlapping sequence shared by fragments V1 and C_R2, it is the most likely amino acid involved in the recognition of MAb 6-15C4. A synthetic peptide (G5-24) which contains the 6-15C4 epitope was then used to study the immunogenic properties of the 6-15C4 epitope.

It was shown that immunoprotection from rabies virus involves a complex interaction of host effectors such as antibody, T lymphocytes, and interferon (8, 22). Since peptide G5-24 contains only a B-cell determinant and not a T-cell epitope of the rabies virus G protein, it is not entirely surprising that mice immunized with this peptide developed only marginal VNA titers (mean titer, 1:9) and were not protected against a lethal rabies virus infection. Mice immunized with the G5-24 peptide coupled to keyhole limpet hemocyanin or bovine serum albumin that developed higher VNA titers (geometric mean titer, 1:30) were also not protected against challenge rabies virus infection. An analysis of the protective immune mechanism induced by rabies virus N protein has previously revealed that both T-helper cells and anti-N protein antibodies are involved in the protective mechanisms against rabies virus challenge (8), suggesting that an efficacious vaccine must condition the immunized animal to respond, when exposed to the virus, by prompt production of virus-specific antibody and virus-specific T cells. In order to provide these conditions, we have covalently linked the G5-24 peptide with the 31-D peptide derived from N protein, which carries a dominant epitope for T-helper cells of the *H-2^k* haplotype (11). Immunization of C3H mice with this tandem peptide resulted not only in the production of VNA but also in significant protection against a lethal rabies virus challenge (Tables 2 and 3). The observation that peptides representing both T- and B-cell determinants are more immunogenic than peptide-carrier protein conjugates is in agreement with a previous report which showed that peptides engineered by colinear synthesis of a B-cell determinant of the major protein VP6 of bovine rotavirus and a T-cell determinant of the influenza virus hemagglutinin were more immunogenic than the corresponding bovine serum albumin-conjugated peptide (2).

Unfortunately, the results of immunization experiments with synthetic peptides do not delineate the exact mechanism responsible for protection against rabies. The induction of rabies virus-specific T-helper cells alone is not sufficient to protect mice against rabies (11). It is also not clear whether the protection conferred by peptide G5-24-31D is solely mediated by VNA. Animals immunized with G5-24-31D were protected against rabies, but these animals produced,

in addition to VNA, rabies virus-immune T_H cells and antibodies directed against RNP. We have already demonstrated that RNP-specific antibodies contribute to the protective immunity against rabies (8). Therefore, the induction of protective immunity mediated by peptide G5-24-31D is most likely the result of an interaction of rabies virus-specific T-helper cells, virus-neutralizing antibodies, and antibodies directed against internal viral RNP.

The immunization experiments with peptide G5-24-31D demonstrate that it is possible to generate a synthetic vaccine against rabies. However, to be efficacious in an outbred population and in different animal species, a synthetic peptide vaccine must contain a T-cell determinant which is immunodominant in many different major histocompatibility complex haplotypes. All rabies virus-specific T-cell determinants described so far are under Ir gene control and therefore cannot be used as reliable immunogens in an outbred population (11, 16).

These results suggest that future research efforts to design a peptide vaccine against rabies should focus on the construction of peptides containing multiple T-cell epitopes that will be recognized by a large variety of genetically distinct hosts.

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LITERATURE CITED

- Atherton, E., H. Fox, D. Harkiss, C. J. Logan, R. C. Sheppard, and B. J. Williams. 1978. A mild procedure for solid phase peptide synthesis: use of fluorenylmethylcarbonyl amino-acids. *J. Chem. Soc. Chem. Commun.* 1978:537-539.
- Borras-Cuesta, F., A. Petit-Camuridan, and Y. Fedon. 1987. Engineering of immunogenic peptides by co-linear synthesis of determinants recognized by band T cells. *Eur. J. Immunol.* 17: 1213-1215.
- Bunschoten, H., M. Gore, I. J. T. M. Claassen, F. G. C. M. UytdeHaag, B. Dietzschold, W. H. Wunner, and A. D. M. E. Osterhaus. 1989. Characterization of a new virus neutralizing epitope that denotes a sequential determinant on the rabies virus glycoprotein. *J. Gen. Virol.* 70:291-298.
- Chou, P. Y., and F. G. Fasman. 1978. Prediction of the secondary structure of proteins from their amino acid sequence. *Adv. Enzymol.* 47:45-148.
- Cox, J. H., B. Dietzschold, and L. G. Schneider. 1977. Rabies virus glycoprotein. II. Biological and serological characterization. *Infect. Immun.* 16:754-759.
- Cox, J. H., B. Dietzschold, F. Weiland, and L. G. Schneider. 1980. Preparation and characterization of rabies virus hemagglutinin. *Infect. Immun.* 30:572-577.
- Dietzschold, B., J. H. Cox, L. G. Schneider, and H. Koprowski. 1977. Isolation and purification of a polymeric form of the glycoprotein of rabies virus. *J. Gen. Virol.* 40:131-139.
- Dietzschold, B., M. Gore, H. Ertl, L. Otvos, Jr., and H. Koprowski. 1989. Analysis of protective immune mechanisms induced by rabies nucleoprotein, p. 295-309. *In* B. W. J. Mahy and D. Kolakofski (ed.), *Genetics and pathogenicity of negative-strand viruses*. Elsevier, New York.
- Dietzschold, B., M. Lafon, H. Wang, L. Otvos, E. Celis, W. H. Wunner, and H. Koprowski. 1987. Localization and immunological characterization of antigenic domains of the rabies virus internal N and NS proteins. *Virus Res.* 8:103-125.
- Dietzschold, B., T. J. Wiktor, R. Macfarlan, and A. Varrichio. 1982. Antigenic structure of rabies virus glycoprotein: ordering and immunological characterization of the large CNBr cleavage fragments. *J. Virol.* 44:595-602.

11. Ertl, H. C. J., B. Dietzschold, M. Gore, L. Otvos, Jr., J. K. Larson, W. H. Wunner, and H. Koprowski. 1989. 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.
12. Glajch, J. L., J. C. Gluckman, J. G. Charikofsky, J. M. Minor, and J. J. Kirkland. 1985. Simultaneous selectivity optimization of mobile and stationary phases in reversed-phase liquid chromatography for isocratic separations of phenylthiohydantoin amino acid derivatives. *J. Chromatogr.* **318**:23-39.
13. Hjerten, S. 1962. Chromatographic separation according to size of macromolecules and cell particles on columns of agarose suspensions. *Arch. Biochem. Biophys.* **99**:466-475.
14. Lafon, M., J. Ideler, and W. H. Wunner. 1984. Investigation of the antigenic structure of rabies virus glycoprotein by monoclonal antibodies. *Dev. Biol. Stand.* **57**:219-225.
15. Lafon, M., T. J. Wiktor, and R. I. Macfarlan. 1983. Antigenic sites on the CVS rabies virus glycoprotein: analysis with monoclonal antibodies. *J. Gen. Virol.* **64**:843-851.
16. Macfarlan, R. I., B. Dietzschold, T. J. Wiktor, M. Kiel, R. Houghton, R. A. Lerner, J. G. Sutcliffe, and H. Koprowski. 1984. T cell responses to cleaved rabies virus glycoprotein and to synthetic peptides. *J. Immunol.* **133**:1748-2752.
17. Prehaud, C., P. Coulon, F. Lefay, C. Thiers, and A. Flamand. 1988. Antigenic site II of the rabies virus glycoprotein: structure and role in viral virulence. *J. Virol.* **62**:1-7.
18. Schaeffer, H., and G. Von Jagow. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **166**:368-379.
19. Seif, I., P. Coulon, P. Rollin, and A. Flamand. 1985. Rabies virulence. Effect on pathogenicity and sequence characterization of rabies virus mutations affecting the antigenic site III of the glycoprotein. *J. Virol.* **53**:926-934.
20. Sikes, R. K., W. F. Cleary, H. Koprowski, T. J. Wiktor, and M. M. Kaplan. 1971. Effective protection of monkeys against death from street virus by post-exposure administration of tissue culture rabies vaccine. *Bull. W.H.O.* **45**:1-11.
21. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350-4354.
22. Turner, G. S. 1985. Immune response after rabies vaccination: basic aspects. *Ann. Inst. Pasteur Virol.* **136E**:453-460.
23. Wiktor, T. J., B. Dietzschold, R. N. Leamson, and H. Koprowski. 1977. Induction and biological properties of defective interfering particles of rabies virus. *J. Virol.* **21**:626-635.
24. Wiktor, T. J., R. I. Macfarlan, C. M. Fogg, and H. Koprowski. 1984. Antigenic analysis of rabies and Mokola virus from Zimbabwe using monoclonal antibodies. *Dev. Biol. Stand.* **57**:199-211.
25. Wiktor, T. J., R. I. Macfarlan, K. J. Reagan, B. Dietzschold, P. J. Curtis, W. H. Wunner, M.-P. Kieny, R. Lathe, J.-P. Lecocq, M. Mackett, B. Moss, and H. Koprowski. 1984. Protection from rabies by a vaccinia virus recombinant containing the rabies virus glycoprotein gene. *Proc. Natl. Acad. Sci. USA* **81**:7194-7198.
26. Wunner, W. H., B. Dietzschold, R. I. Macfarlan, C. L. Smith, E. Golub, and T. J. Wiktor. 1985. Localization of immunogenic domains on the rabies virus glycoprotein. *Ann. Virol.* **136E**:353-362.
27. Wunner, W. H., B. Dietzschold, C. L. Smith, M. Lafon, and E. Golub. 1985. Antigenic variants of CVS rabies virus with altered glycosylation sites. *Virology* **140**:1-12.
28. Wunner, W. H., C. L. Smith, M. Lafon, J. Ideler, and T. J. Wiktor. 1984. Comparative nucleotide sequence analysis of the glycoprotein gene of antigenically altered rabies viruses, p. 279-284. In R. W. Compans and D. H. L. Bishop (ed.), *Nonsegmented negative-strand viruses: arenaviruses, bunyaviruses and orthomyxoviruses*. Academic Press, Inc., Orlando, Fla.