Induction of Rabies Virus-Specific T-Helper Cells by Synthetic Peptides That Carry Dominant T-Helper Cell Epitopes of the Viral Ribonucleoprotein

H. C. J. ERTL,* B. DIETZSCHOLD, M. GORE, L. OTVOS, JR., J. K. LARSON, W. H. WUNNER,
AND H. KOPROWSKI

The Wistar Institute of Anatomy and Biology, 3601 Spruce Street, Philadelphia, Pennsylvania 19104-4268

Received 12 December 1988/Accepted 14 March 1989

The T-helper cell response to the internal proteins of rabies virus was investigated. The rabies virus nucleoprotein was shown to be a major target antigen for T-helper cells that cross-react between rabies and rabies-related viruses. T-helper cells were assayed in vitro by testing virus-induced lymphocytes for lymphokine secretion in response to antigen. Immunodominant T-helper cell epitopes of the viral nucleoprotein were identified in vitro by using synthetic peptides delineated from the amino acid sequence of the nucleoprotein. The response to synthetic peptides was under Ir gene control. Antigenic peptides were tested in vivo for stimulation of rabies virus-specific T-helper cells. Inoculation of mice with peptides bearing immunodominant T-helper cell epitopes resulted in an accelerated and enhanced neutralizing antibody response upon booster immunization with inactivated rabies virus.

Rabies virus, a rhabdovirus of the genus *Lyssavirus*, has five viral structural proteins: a glycoprotein (G protein), a membrane protein (M protein), a large protein (L protein), a nominal phosphoprotein (NS protein), and a nucleoprotein (N protein). The internal proteins, i.e., the L, N, and NS proteins, are complexed with the viral RNA to form the viral ribonucleoprotein (RNP). The rabies virus G protein, which is the only antigen able to induce a virus-neutralizing antibody (VNA) response (5), was considered responsible for the induction of a protective immune response to rabies virus. Recently, it has been demonstrated that RNP can induce a protective immune response in both mice and raccoons against a subsequent peripheral challenge with rabies virus (10). RNP-immunized mice showed an augmented and accelerated VNA response upon booster immunization with homologous and heterologous rabies viruses, indicating that internal rabies virus proteins induce T-helper cells for B-cell activation. In contrast to the rabies virus G protein, which shows a high degree of antigenic variability among different fixed and street rabies virus strains (8, 11), the N protein is more conserved, as was shown by using panels of monoclonal antibodies (7), and might therefore be more useful for the design of a subunit or synthetic vaccine.

The mechanisms by which vaccination with rabies virus RNP confers protection against a subsequent challenge with virus are not understood. As a first step in investigating the immune responses induced by RNP, the T-helper cell response to rabies virus internal proteins was studied. We identified the N protein, the predominant protein of the rabies virus RNP complex, as a major antigen responsible for the stimulation of lymphokine-secreting T-helper cells that cross-react with different rabies and rabies-related viruses. To identify T-helper cell epitopes, 40 overlapping synthetic peptides covering nearly the entire amino acid sequence of the N protein were tested for induction of lymphokine secretion from rabies virus-primed T cells derived from inbred mice of different genetic backgrounds. The use of major histocompatibility complex-recombinant mice

defined the role of Ir genes in controlling T-cell responses to single N-protein epitopes. Peptides that were shown to carry dominant T-helper cell epitopes were used in vivo for stimulation of a rabies virus-specific T-helper cell response. We investigated the effect of peptide priming on the development of VNA titers after a booster immunization with virus and the effect of challenge with live rabies virus on mouse mortality rates.

MATERIALS AND METHODS

Animals. Female C3H/He (*H-2^k*), B10.BR (*H-2^k*), B10.A (*H-2^a*), C57BL/6 (*H-2^b*), C3H.SW (*H-2^b*), BALB/c (*H-2^d*), DBA/2 (*H-2^d*), and B10.D2 (*H-2^d*) mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and used at the age of 6 to 10 weeks. Male and female Lewis rats were purchased from Charles River Breeding Laboratories, Inc., Wilmington, Mass., and used at the age of 3 months.

Viruses and antigens. The rabies virus strain Evelyn-Rokitnicki-Abelseth (ERA) and challenge virus strain 11 (CVS-11) and the rabies-related strains Mokola 3 and Duvenhage 6 were propagated on BHK-21 cells as described previously (23). Viruses were purified as described previously (23), suspended in phosphate-buffered saline, inactivated with β -propiolactone (BPL), and adjusted to a protein concentration of 100 μ g/ml. Viral RNP was isolated as described previously (19). The structural proteins, i.e., G, M, N, and NS proteins of rabies virus, were purified by polyacrylamide gel electrophoresis as recently described (7).

Cell lines. The lymphokine (interleukin-2 and interleukin-4)-dependent HT-2 cell line was maintained in vitro in Dulbecco modified Eagle (DME) medium supplemented with 10% fetal bovine serum (FBS) and 5% rat concanavalin A (ConA) supernatant (RCAS). Mixed leukocyte culture (MLC) cells were generated by culturing 5×10^7 C57BL/6 splenocytes with 10^8 gamma-irradiated DBA/2 splenocytes for 10 to 14 days in 40 ml of DME medium supplemented with 2% FBS (18). BHK-21 cells were maintained as described previously (10).

Rat ConA supernatant. Rat splenocytes (1.2×10^6 /ml) were cultured for 48 h with ConA (2.5 μ g/ml) in DME

* Corresponding author.

1	MDADKIVFKVNNQVSLKPEIIVDQHEYKYPKIDKKPCITLKGAPDLNKAYKSVLSGMSAAKLDPPDDV				
71	CSYLAAMQFFEGTCDPWTSGYIVGIARKGDKITPGSLVEIKRTDVEGNWALTGGMELTRDPTVPEHASL	a	b		
141	VGLLSLYRLSKISGQNTGNYKTNIADRIEQIFETAPFVKIVEHHTLMTTHKMCANWSTIPNFRFLAGTY	1D	1- 15;	11D	101-115;
211	DMFFSRIEHLYSAIRVGTVTAYEDCSGLVSFTGFIQINLTVREAILYFFHKNFEEIIRMFEPGQETA	2D	11- 25;	12D	111-125;
281	VPHSYFIHFRSLGLSGKSPYSSNAVGHVFNLIHFVGCYMGQVRSINATVIAACAPHEMSVLGGYLGEFF	3D	21- 35;	13D	121-135;
351	GKGTFFRRFDEKELQEYEAELTKTDVALADDGTVNSDDEDFSGETRSPEAVYTRIMNGGRLKRSH	4D	31- 45;	14D	131-145;
421	IRRYVSVSSNHQARPNFAEFLNKTYSSDS	5D	41- 55;	15D	141-155;
		6D	51- 65;	16D	151-165;
		7D	61- 75;	17D	161-175;
		8D	71- 85;	18D	171-185;
		9D	81- 95;	19D	181-195;
		10D	91-105;	20D	191-205;
				21D	201-215;
				22D	211-225;
				23D	221-235;
				24D	231-245;
				25D	241-255;
				26D	251-265;
				27D	261-275;
				V12b1	269-283;
				V12b2	279-293;
				V12b3	288-303;
				V12b4	299-303;
				V12b	313-338;
				28D	343-358;
				29D	351-368;
				V10c	369-383;
				30D	394-408;
				31D	404-418;
				32D	414-428;
				33D	424-438;
				34D	434-450.

FIG. 1. Sequence of ERA N protein shown in single-letter code (left) and alignment of synthetic peptides with the amino acid sequence of the ERA N protein (right). a, Designation of peptides; b, position of peptides within the ERA N protein sequence.

supplemented with 2% FBS. Supernatants were harvested, adsorbed twice with Sephadex G-25 beads (0.2 g/100 ml) to remove residual ConA, sterile filtered, and stored at -20°C .

Deduced amino acid sequence of the rabies virus ERA N protein. The amino acid sequence of the ERA N protein was deduced from the nucleotide-coding sequence of the N gene of genomic RNA. Synthetic oligodeoxyribonucleotides were used to prime the synthesis of complementary DNA transcripts of the genomic RNA in the presence of dideoxynucleoside triphosphates as described previously (24). DNA primers were synthesized on a DNA synthesizer (model 380A; Applied Biosystems Inc., Foster City, Calif.) and purified by reverse-phase high-pressure liquid chromatography on a Beckman OD5 Ultrasphere column as described previously (24). The N-protein nucleotide-coding sequence was determined from the 3' end by using a synthetic DNA primer that hybridized to bases 48 to 69 of the genome RNA. These include the 5' end of the leader sequence and first 11 bases of noncoding sequences at the 3' end of the N gene upstream from the coding sequence for N protein. The entire nucleotide-coding sequence of the N gene was determined in a stepwise manner with additional DNA primers derived from subsequently determined downstream sequences. Translation of the coding sequence of the longest open reading frame in the complementary DNA (corresponding to the mRNA transcript), which is initiated with the first ATG and terminates with translation stop codon TAA 1,351 to 1,353 nucleotides downstream, corresponds to a protein of 55,500 daltons (11). The amino acid sequence of the ERA N protein is shown in single-letter code in Fig. 1 (left) and has the same amino acid length as the one for the Pasteur virus (PV) strain N protein reported by Tordo et al. (22).

Preparation of synthetic peptides. Peptides 1D, 3D, 31D, 32D, 33D, 34D, V12b, and V10c were synthesized on *p*-alkoxy-benzylalcohol-resin by using *N*-fluorenylmethoxycarbonyl-amino acids (1, 15). After cleavage of the solid support, peptides were dialyzed in cutoff tubing (MWCO 1000; Spectrum, Los Angeles, Calif.) overnight. The composition of the peptides was verified by amino acid analysis. The other peptides were synthesized by BioSearch, San Rafael, Calif., by using multiple-peptide synthesis (16). All peptides were linked at the amino-terminal end to palmitic acid. A Lys-Gly-Gly bridge was added as a spacer to facilitate the binding of two palmitic acid molecules (14). Although the spacer and the two attached palmitic acid molecules were not essential for the peptides to induce a primary response in vivo or to elicit a secondary response in vitro, peptides coupled to palmitic acid, as was tested for peptide 31D, induced a better response in vivo and in addition could be used at lower concentrations to elicit an optimal response in vitro (data not shown). The position of the individual peptides within the ERA N protein sequence is shown in Fig. 1 (right).

Immunization of mice. Groups of inbred mice were injected subcutaneously (s.c.) in both hind legs with 10 μl of the antigen solution, i.e., 1 μg of BPL-inactivated ERA virus in saline or 5 μg of synthetic peptide in complete Freund adjuvant (CFA). Mice were sacrificed 6 to 7 days later, draining lymph nodes (i.e., popliteal lymph nodes) were harvested, and single-cell suspensions were prepared. In one experiment, mice were immunized intraperitoneally (i.p.) with 1 μg of BPL-inactivated ERA virus in saline, and their splenocytes were used as responder cells 1 week later. Mesenteric lymph nodes from naive mice and popliteal lymph nodes from mice injected with CFA were treated identically and used as negative controls. In some experiments, mesenteric lymph node lymphocytes from mice that had been immunized 6 to 7 days earlier were used as negative controls. These cells had been shown to completely lack a T-cell response to rabies virus or rabies virus antigens. To test the efficacy of peptide priming on stimulation of B cells upon booster immunization, mice were inoculated i.p. with 20 μg of synthetic peptides in CFA or with CFA only and injected i.p. 4 weeks later with 1 μg of BPL-inactivated ERA virus. To test for mortality, groups of 10 mice were immunized i.p. with 5 μg of ERA RNP in CFA, 10 μg of peptide in CFA, or CFA only. Mice were challenged 4 weeks later intramuscularly (i.m.) in the hind leg with 10 mouse i.m. 50% lethal doses (corresponding to $\sim 10^7$ intracerebral 50% lethal doses) of CVS-11. With regard to residues 404 to 418 (peptide 31D), CVS-11 has the same sequence as ERA (25); accordingly, ERA-induced T-cell clones specific for these residues cross-react with CVS-11 (H. Ertl, unpublished data).

T-helper cell assay. Lymphocytes (2×10^5) derived from lymph nodes or spleens of immune (or naive) mice were cultured in 150 μl of DME supplemented with 2% FBS in wells of a round-bottom microdilution plate with BPL-inactivated virus, viral protein, or synthetic peptide. The antigen concentrations that were shown to induce optimal stimulation of long-term T-helper cells to rabies virus were used throughout the experiments (see figure legends). Supernatants were harvested 20 to 24 h later and tested for induction of proliferation of a lymphokine-dependent T-cell line as follows: 2×10^3 HT-2 cells or 1×10^4 MLC cells in 100 μl of DME supplemented with 10% (for HT-2 cells) or 2% (for MLC cells) FBS were cultured in triplicate with 50 μl of either concentrated or diluted supernatant, fresh medium (negative control), or serial dilutions of rat ConA supernatant (positive control). Even at the highest concentration tested, the supernatants derived from stimulated lymphocytes did not contain sufficient amounts of lymphokines to cause maximal activation of the indicator cells as was estimated in each experiment by using serial dilutions of rat ConA supernatant. Proliferation was measured 40 to 48 h later by a 6-h [^3H]thymidine pulse (0.5 μCi per well).

FIG. 2. Specificity of rabies virus-induced T-helper cells. Groups of mice were immunized with 1 μ g of ERA BPL s.c., and draining lymph node lymphocytes or mesenteric lymphocytes (data shown for C3H control) were cocultured 7 days later with purified viral proteins (10 μ g/ml). Culture supernatants were harvested 24 h later and tested at 1/3 (■) and 1/6 (▨) dilutions on 2×10^3 HT-2 cells (C3H anti-ERA [I], C3H control, BALB/c and C57BL/6 anti ERA) or on 1×10^4 C57BL/6 anti DBA/2 lymphocytes (C3H anti ERA [II]). Indicator cells were pulsed 42 to 44 h later for 6 h with [3 H]thymidine, harvested, processed, and counted. Data are expressed as the mean $\pm$ standard deviations of triplicate determinations. Results were obtained from three separate experiments.

Determination of virus-neutralizing antibody titers. Blood was collected from mice 5 and 10 days after challenge. Fivefold dilutions of sera were incubated with ERA virus and subsequently titrated on BHK-21 cells as described previously (9). The reciprocal of the serum dilution resulting in a 50% reduction in the number of infected cells was considered the neutralization titer.

Comparison of amino acid sequences. The sequences of some of the synthetic peptides carrying immunodominant epitopes were screened for homology with known protein sequences by using the protein sequence data base PIR (12).

Secondary-structure prediction. The prediction of the secondary structure of the immunodominant epitopes was made by using the Hydrocalc program (Advanced Chemtech).

RESULTS

Antigen specificity of rabies virus-induced T-helper cells. Initial experiments were designed to determine the relative role of different rabies virus proteins in the T-helper cell response to rabies virus. T-helper cell activation was tested by measuring the release of lymphokines, a method which in this system was found to be more sensitive than testing antigen-mediated proliferation of lymphocytes (H. Ertl, unpublished data). All the rabies virus proteins tested induced secretion of lymphokines from ERA-immune lymphocytes (Fig. 2) as measured by thymidine incorporation by lymphokine-dependent indicator cells (Table 1). As expected, intact virus induced the highest release of lymphokines. Independent of the host (i.e., different mouse strains or rabbit; data for rabbit not shown), the response to purified viral proteins usually showed the following order of potency: ERA RNP > ERA N > ERA G > ERA NS > ERA M. These data suggest that the N protein of rabies virus is a major target antigen for rabies virus-specific T-helper cells.

Cross-reactivity of ERA RNP-specific T-helper cells between different rabies and rabies-related virus strains. Although internal RNPs are the most conserved antigens among rabies

and rabies-related viruses, some variability that might affect T-helper cell epitopes has been demonstrated (7). To determine whether RNP-specific T-helper cells cross-reacted with the RNP or N protein from different rabies or rabies-related viruses, BALB/c mice were immunized with ERA BPL s.c., and draining lymph node lymphocytes were tested 7 days later for lymphokine release in response to RNP or N protein derived from rabies or rabies-related viruses. Significant cross-reactivity was found between the rabies virus strains ERA and CVS and between the rabies-related Duvenhage and Mokola viruses, although the latter reactivity was less pronounced (Fig. 3).

Identification of dominant T-helper cell epitopes of the ERA N protein. To determine dominant T-helper cell epitopes of the ERA N protein, inbred mice of different *H-2* haplotypes were immunized with ERA BPL s.c., and draining lymph node lymphocytes were tested for lymphokine release in response to each of the 40 overlapping synthetic peptides (Fig. 1) homologous to nearly the entire sequence of the ERA N protein. Whether different peptides elicited a re-

TABLE 1. Identification of lymphokines

Responder cells ^a	Anti-body ^b	Thymidine incorporation (cpm) $\pm$ SD		
		No antigen	ERA BPL	Peptide 31 ^c
B10.BR anti-ERA	None	308 $\pm$ 48	3,466 $\pm$ 235	834 $\pm$ 149
B10.BR anti-ERA	11B11	352 $\pm$ 40	3,238 $\pm$ 154	818 $\pm$ 144
B10.BR anti-31	None	130 $\pm$ 33	817 $\pm$ 22	570 $\pm$ 87
B10.BR anti-31	11B11	170 $\pm$ 33	649 $\pm$ 103	537 $\pm$ 21
B10.A anti-ERA	None	128 $\pm$ 21	2,042 $\pm$ 145	559 $\pm$ 67
B10.A anti-ERA	11B11	170 $\pm$ 27	1,768 $\pm$ 75	459 $\pm$ 30
B10.A anti-31	None	146 $\pm$ 76	812 $\pm$ 30	437 $\pm$ 17
B10.A anti-31	11B11	220 $\pm$ 131	761 $\pm$ 105	579 $\pm$ 155

^a Origin of immune lymphocytes.

^b 11B11 did not inhibit proliferation of HT-2 cells in response to recombinant interleukin-2. The response to recombinant interleukin-4 was inhibited by 97%.

^c Antigen used for in vitro restimulation.

FIG. 3. Cross-reactivity of ERA RNP-specific T-helper cells between different rabies and rabies-related viruses. Groups of BALB/c mice were immunized with 1 μ g of ERA BPL s.c., and draining lymph node lymphocytes and mesenteric lymph node lymphocytes were restimulated 7 days later with one of the following (10 μ g/ml): ERA BPL virus, RNP or N protein derived from rabies virus strain ERA or CVS-11, or the rabies-related viruses Mokola 3 or Duvenhage 9. Supernatants were harvested 24 h later and tested at a 1/3 (■) or 1/6 (▨) dilution on HT-2 cells. Control lymphocytes showed no significant release of lymphokines upon coculture with antigen (data not shown).

sponse from ERA-immune lymphocytes depended on the haplotype of the lymphocyte donor. The N-protein-specific T-helper cell response of mice of the *H-2^k* and *H-2^a* haplotype was clearly dominated by a single epitope expressed on peptide 31D (Fig. 4A); lymphocytes of the *H-2^b* haplotype responded to two epitopes expressed on peptides 3D and 30D (Fig. 4B). The reactivity profile as tested in C57BL/6 mice was not restricted to draining lymph node lymphocytes; splenocytes of mice immunized i.p. responded to precisely the same epitopes. Rabies virus-immune lymphocytes of *H-2^d* mice had a more complex response to N-protein peptides (Fig. 4C). None of the peptides carried a clearly dominant epitope for immune *H-2^d* lymphocytes, but a number of peptides (2D, 3D, 10D, and 31D) induced rather low responses.

Neither the peptides nor any of the viral proteins tested elicited a measurable lymphokine response from any of the control lymphocytes.

Immunogenicity of ERA N peptides. Peptides shown to stimulate a T-helper cell response of ERA virus-immune lymphocytes in vitro were tested in vivo for stimulation of rabies virus-specific T-helper cells. Mice of different inbred strains were immunized with peptides in CFA. Draining lymph node lymphocytes were tested 6 to 7 days later for lymphokine release in response to whole virus or viral proteins derived from different rabies or rabies-related viruses. Lymphocytes were also tested for a response to the peptide used for immunization, as well as to the two adjacent overlapping peptides and unrelated peptides. In C3H mice, peptide 31D-induced T-helper cells that responded not only to the homologous peptide 31D but also to ERA virus, ERA N, and ERA RNP (Fig. 5A). Peptide 31D-immune lymphocytes did not respond to other rabies virus proteins such as G, NS, or M or to any other peptides tested. Control peptides such as 8D failed to induce a measurable T-helper cell response.

C57BL/6 mice responded to peptide 3D (and peptide 30D [data not shown]) but failed to respond to peptides 31D (Fig. 5B). Peptide 3D induced RNP-specific T-helper cells that cross-reacted with rabies and rabies-related viruses.

Three immunogenic peptides were identified in BALB/c mice, i.e., 3D, 10D, and 31D. Although these three peptides

induced T-helper cells which showed responses upon re-stimulation with the homologous peptide, the response to whole virus or viral RNP was marginal. With peptides 3D and 31D, ERA RNP-specific responses were observed only when high concentrations of ERA RNP were used for in vitro restimulation. Peptides 13D (Fig. 5C), 8D, and 22D (data not shown) failed to induce a measurable response in BALB/c mice.

The in vivo effects of peptides that carry dominant epitopes were evaluated. Neither of the peptides shown to carry immunodominant T-helper cell epitopes (i.e., peptide 3D in C57BL/6 mice and 31D in C3H mice) induced an antibody response to N protein (data not shown). Upon immunization with BPL-inactivated ERA virus, peptide (plus CFA)-primed mice responded within 5 days with VNA titers that were significantly enhanced compared with titers of control mice that had been primed with CFA only. The difference was even more pronounced 10 days after challenge (Fig. 6). In addition, VNA titers in peptide 31D-primed C3H mice and peptide 3D-primed C57BL/6 mice were enhanced upon immunization with BPL-inactivated Duvenhage virus (data not shown). Consistent with the marginal rabies N-protein-specific T-helper cell response in vitro, no significant increase of VNA titers was observed in BALB/c mice primed with peptide 31D.

The accelerated and enhanced B-cell response to rabies virus G protein observed in peptide-primed and virus-challenged mice was comparable in magnitude to the response obtained with RNP priming followed by viral challenge, which had previously been shown to induce protective immunity (10). But when C3H mice immunized with peptide 31D (in CFA), RNP (in CFA), or CFA only were challenged peripherally with a lethal dose of CVS-11 rabies virus, which has the identical amino acid sequence in residues 404 to 418 (i.e., peptide 31D [J. K. Larson, unpublished observation]) as the ERA strain (Fig. 7), no increase in survival could be observed in peptide-primed mice.

DISCUSSION

The goal of this study was to define the T-helper cell response to the rabies virus N protein. The T-helper cell response to rabies virus internal proteins has been shown to induce protective immunity to a challenge with homologous and heterologous rabies viruses (10). We have identified the viral N protein as a major target antigen for murine T-helper cells that cross-react with RNP and N proteins derived from different fixed rabies virus strains (CVS and PM; data for PM not shown) as well as rabies-related viruses (Duvenhage and Mokola). Comparable results have been published for proliferating T lymphocytes derived from human vaccinees (3).

To identify immunodominant sites of the rabies virus N protein, 40 overlapping peptides covering the ERA N-protein sequence were synthesized and tested for their ability to elicit lymphokine release with ERA virus-induced T-helper cells derived from several mouse strains. The reactivity pattern of these peptides was linked to determinants encoded by the genes of the major histocompatibility complex. One dominant epitope expressed on peptide 31D was identified in mice of the *H-2^k* and *H-2^a* haplotype, while two epitopes expressed on peptides 30D and 3D elicited a response from virus-immune T-helper cells of the *H-2^b* haplotype. The epitopes expressed on these immunodominant peptides are presumably not the only epitopes of the rabies virus N protein that are recognized by virus-induced T-helper cells of the *H-2^k* and *H-2^a* or *H-2^b* haplotypes. Other

FIG. 4. Response of ERA-immune T-helper cells to ERA N peptides. Groups of mice were injected with 1 μ g of ERA BPL s.c., and 2×10^5 lymphocytes from draining lymph nodes, as well as control lymphocytes (mesenteric lymph nodes, data not shown), were cocultured 7 days later with 1 μ g of virus or peptide per ml. After 24 h, supernatants were harvested and tested on HT-2 cells. Shown are epitopes of rabies virus N proteins *H-2^k* (A), *H-2^b* (B), and *H-2^d* (C).

minor epitopes (i.e., epitopes that are recognized by too low a percentage of the total pool of ERA N-protein-specific T-helper cells to be detected by the assay system used) have been identified in our laboratory by long-term T-cell lines tested with the same panel of peptides by using the same donor mouse strains (H. Ertl, unpublished data). Furthermore, the synthetic peptides that were used overlapped by only five amino acids and thus might not carry every single T-cell epitope present on the N protein. Although lymphocytes of BALB/c and B10.D2 origin responded to peptide 31D (the immunodominant peptide in *H-2^k* and *H-2^a* haplotypes [that both express *K^k* and *I^k* determinants]) and peptide 3D (one of the immunodominant peptides in *H-2^b* haplotypes), none of the 40 ERA N peptides was shown to express a clearly dominant epitope for T-helper cells of the *H-2^d* haplotype. In the converse experiments, peptides that carried immunodominant epitopes were found to induce a strong T-helper response in mice of the appropriate haplo-

type, as determined by the secretion of lymphokines to antigen in vitro or by the augmented VNA response upon booster immunization with virus in vivo.

The rabies virus N protein is the most antigenically conserved of the rabies virus structural proteins, as was determined by monoclonal antibodies. So far the nucleotide sequences of N proteins of only two laboratory virus strains, i.e., ERA and PV (22), both initially isolated from domestic animals, have been established. Four amino acid exchanges were found between the deduced amino acid sequences of the N protein of the ERA and the PV strains of rabies virus (reviewed in reference 25). Two of these amino acid exchanges (i.e., residue 26, His_{ERA} to Tyr_{PV}; residue 410, Met_{ERA} to Ile_{PV}) are located within the sequence of two of the peptides carrying immunodominant epitopes, i.e., peptides 3D and 31D, respectively, neither of which expresses any of the known B-cell epitopes. Considering the length of the peptides (15 amino acids), it is possible that the amino acid exchanges occurred among residues that are not part of the T-cell epitope. It has been shown previously that T cells preferentially recognize variable regions of protein pathogens; for example, murine cytolytic T cells to the human immunodeficiency virus respond predominantly to a highly variable sequence of the viral glycoprotein (21). Similarly, human T cells specific for the circumsporozoite protein of *Plasmodium falciparum* have been demonstrated to recognize immunodominant epitopes in the polymorphic regions of the molecule (13). These data might suggest that T cells exert selective pressure on microbial antigens, such as by interrupting virus multiplication by direct lysis of infected

FIG. 5. Induction of rabies virus-specific T-helper cells by synthetic peptides of the viral N protein. Mice (C3H [A], BALB/c [B], C57BL/6 [C]) were injected s.c. with 5 μ g of peptide in CFA. After 7 days, draining lymph node lymphocytes were harvested and 2×10^5 lymphocytes were restimulated with virus, viral protein, or peptide (1 μ g/ml; 10 $\times$ RNP = 10 μ g/ml). Supernatants were harvested 24 h later and tested on HT-2 cells.

FIG. 6. Effect of priming with synthetic peptides on VNA titers. Groups of 10 mice were injected with 20 μ g of peptide in CFA i.p. Mice were bled individually 5 and 10 days later, and VNA titers to ERA virus were determined as described previously (14).

cells or by release of lymphokines such as interferon or leucotoxin.

Comparison of the amino acid sequences of peptide 3D and peptide 31D with those of other protein sequences (12) revealed no significant homology with any known protein. Both peptides 3D and 31D had hydrophilic regions (positions 5 through 12 for 3D and positions 6 through 12 for peptide

FIG. 7. Survival of C3H mice immunized with peptide 31D after i.m. challenge with rabies virus. Groups of 10 C3H mice were immunized i.p. with 5 μ g of ERA RNP in CFA (●), 10 μ g of peptide 31D in CFA (□), or CFA only (■). Four weeks after immunization, mice were challenged i.m. in the hind leg with 10 $\times$ mouse i.m. 50% lethal dose (corresponding to $\sim 10^7$ intracerebral 50% lethal doses). Mice were observed for 21 days for paralysis or death.

31D). The predicted secondary structure of 3D suggests an alpha helix (residues 2 through 7) followed by a possible β turn (residues 8 through 11) and a random coil (residues 12 through 15), while 31D presumably has a β sheet at the N terminus (residues 1 through 6) followed by a β turn (residues 7 through 10) and an alpha helix (residues 11 through 14) (4). Although peptide 31D seems to have a strong helical moment, neither of the two peptides is likely to form an amphipathic alpha helix as has been predicted for potential T-cell recognition sites (6, 20), strongly suggesting that an amphipathic helix is not a prerequisite for a T-cell epitope.

Although the ERA N peptides bearing immunodominant T-helper cell epitopes induced a significant T-helper cell response in vivo and an accelerated VNA response upon booster immunization with virus, peptide-immune mice died upon a subsequent challenge with live rabies virus. Thus, neither the peptide-induced T-helper cell response nor the increase in VNA titer upon antigenic boost was sufficient to protect against a lethal challenge in mice. Depending on the virus strain, the site of inoculation, and the age and species of the host, rabies viruses enter the nervous system on the average within 24 to 48 h, then migrate within the long nerves to the central nervous system (reviewed in reference 2) and there cause, in the mouse model described here, clinical symptoms 6 to 10 days after infection. Passively transferred neutralizing antibodies have to be given within 2 h after inoculation with live virus to prevent a lethal infection (Dietzschold et al., unpublished data). In the results presented here, an augmented VNA response was demonstrated 5 days after booster immunization with inactivated virus, which is presumably too late to affect the virus spread into the central nervous system. The protection we observed previously upon vaccination with rabies virus RNP (10) thus cannot be attributed to the accelerated and increased response of neutralizing antibodies but is caused by stimulation of RNP-specific B cells or cytolytic T cells or most likely a complex interaction of humoral as well as cellular immune mechanisms (17).

Nevertheless, a mixture of immunodominant peptides that cause an augmentation of neutralizing antibodies upon booster immunization might be useful for priming and could be followed by low doses of conventional vaccine. This would substantially lower the cost for vaccination of humans with tissue-culture-grown rabies vaccines, which are unaffordable in many countries where rabies virus poses a serious public health threat.

ACKNOWLEDGMENTS

We thank Nancy Ricalton, Georgia Kivulka, and Andrew Lanutti for excellent technical assistance, E. Heber-Katz for helpful discussion, and M. Hoffman (editor) for help in preparing the manuscript.

This work was supported by Public Health Service grants AI 09706-16, AI 18883-06, and AI 23503-02 from the National Institute of Allergy and Infectious Diseases.

ADDENDUM

The deduced amino acid sequence of the ERA N protein reported here and the PV N protein amino acid sequence published by Tordo et al. (22) differ by 4 amino acid residues. This report corrects a previous reference to 5 amino acid differences between the two protein sequences (25). Peptides 25D and 26D were synthesized according to the previously deduced ERA N protein sequence which included amino acid difference 5 (valine for alanine) at residue 253.

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 fluorenylmethoxycarbonyl-amino-acids. *J. Chem. Soc. Chem. Commun.* 1978:537-539.
- Baer, G. M. 1975. Pathogenesis to the central nervous system, p. 181-273. *In* G. M. Baer (ed.). *The natural history of rabies*, vol. 1. Academic Press, Inc., New York.
- Celis, E., R. W. Miller, T. J. Wiktor, B. Dietzschold, and H. Koprowski. 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.
- Chou, D. Y., and G. D. Fasman. 1978. Empirical predictions of protein conformation. *Annu. Rev. Biochem.* 47:251-276.
- Cox, J. H., B. Dietzschold, and L. G. Schneider. 1977. Rabies virus glycoprotein. II. Biological and serological characterization. *Infect. Immun.* 16:754-759.
- DeLisi, C., and J. A. Berzofsky. 1985. T cell antigenic sites tend to be amphipathic structures. *Proc. Natl. Acad. Sci. USA* 82:7048-7052.
- Dietzschold, B., M. Lafon, H. Wang, L. Otvos, Jr., E. Celis, W. Wunner, and H. Koprowski. 1987. Localization and immunological characterization of antigenic domains of the rabies virus internal N and NS proteins. *Virus Res.* 8:1-25.
- Dietzschold, B., C. E. Rupprecht, M. Tollis, M. Lafon, J. Mattei, T. J. Wiktor, and H. Koprowski. 1988. Antigenic diversity of the glycoprotein and nucleocapsid proteins of rabies and rabies-related viruses: implications for epidemiology and control of rabies. *Rev. Infect. Dis.* 10:S785-S798.
- Dietzschold, B., M. Tollis, M. Lafon, W. H. Wunner, and H. Koprowski. 1987. Mechanisms of rabies virus neutralization by glycoprotein-specific monoclonal antibodies. *Virology* 161:29-36.
- Dietzschold, B., H. Wang, C. E. Rupprecht, E. Celis, M. Tollis, H. Ertl, E. Heber-Katz, and H. Koprowski. 1988. Induction of protective immunity against rabies by immunization with rabies virus ribonucleoprotein. *Proc. Natl. Acad. Sci. USA* 84:9165-9169.
- Dietzschold, B., T. J. Wiktor, W. H. Wunner, and A. Varrichio. 1983. Chemical and immunological analysis of the rabies soluble glycoprotein. *Virology* 124:330-337.
- George, D. C., W. C. Baker, and L. T. Hunt. 1986. The protein identification resource. *Nucleic Acids Res.* 14:11-15.
- Good, M. F., D. Pombo, I. A. Quakyi, E. M. Riley, R. A. Houghten, A. Menon, D. W. Alling, J. A. Berzofsky, and L. H. Miller. 1988. Human T cell recognition of the circumsporozoite protein of *Plasmodium falciparum*: immunodominant T cell domains map to the polymorphic regions of the molecule. *Proc. Natl. Acad. Sci. USA* 85:1199-1203.
- Hopp, T. P. 1984. Immunogenicity of a synthetic HBsAg peptide: enhancement by conjugation to a fatty acid carrier. *Mol. Immunol.* 21:13-16.
- Houghten, R.-A. 1985. General method for the rapid solid-phase synthesis of large numbers of peptides: specificity of antigen-antibody interaction at the level of individual amino acids. *Proc. Natl. Acad. Sci. USA* 82:5131-5135.
- Otvos, L., Jr., B. Dietzschold, and L. Krifaludy. 1987. Solid-phase peptide synthesis using tert.-butoxycarbonylamino acid pentafluorophenyl esters. *Int. J. Pept. Protein Res.* 30:511-514.
- Prabhakar, B. S., H. R. Fischman, and N. Nathanson. 1981. Recovery from experimental rabies by adoptive transfer of immune cells. *J. Gen. Virol.* 56:25-31.
- Ryser, J. E., J. C. Cerottini, and K. T. Brunner. 1978. Generation of cytolytic T lymphocytes in vitro. IX. Induction of secondary CTL responses in primary long-term MLC by supernatants from secondary MLC. *J. Immunol.* 120:370-377.
- Schneider, L. G., B. Dietzschold, R. E. Dierks, W. Matthaeus, P.-J. Enzmann, and K. Strohmaier. 1973. Rabies group-specific ribonucleoprotein antigen and a test system for grouping and typing for rhabdoviruses. *J. Virol.* 11:748-755.
- Spouge, J. L., H. R. Guy, J. L. Cornette, H. Margalit, K. Cease, J. A. Berzofsky, and C. DeLisi. 1987. Strong conformational propensities enhance T cell antigenicity. *J. Immunol.* 138:204-212.
- Takahashi, H., J. Cohen, A. Hosmalin, K. B. Cease, R. Houghten, J. L. Cornette, C. DeLisi, B. Moss, R. N. Germain, and J. A. Berzofsky. 1988. An immunodominant epitope of the human immunodeficiency virus envelope glycoprotein gp160 recognized by class I major histocompatibility complex molecule-restricted murine cytotoxic T lymphocytes. *Proc. Natl. Acad. Sci. USA* 85:3105-3109.
- Tordo, N., O. Poch, A. Ermine, and G. Keith. 1986. Primary structure of leader RNA and nucleoprotein genes of the rabies genome: segmented homology with VSV. *Nucleic Acids Res.* 14:2671-2683.
- Wiktor, T. J., B. Dietzschold, R. N. Leamnson, and H. Koprowski. 1977. Induction and biological property of defective interfering particles of rabies virus. *J. Virol.* 21:626-635.
- 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.
- Wunner, W. H., J. K. Larson, B. Dietzschold, and C. L. Smith. 1988. The molecular biology of rabies viruses. *Rev. Infect. Dis.* 10:S771-S789.