Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 7194–7198, November 1984
Immunology

Protection from rabies by a vaccinia virus recombinant containing the rabies virus glycoprotein gene

(live and inactivated vaccines/cytotoxic T lymphocytes)

TADEUSZ J. WIKTOR*, RODERICK I. MACFARLAN*, KEVIN J. REAGAN*, BERNHARD DIETZSCHOLD*, . . PETER J. CURTIS*, WILLIAM H. WUNNER*, MARIE-PAUL KIENY†, RICHARD LATHE†§, JEAN-PIERRE LECOCQ†, MICHAEL MACKETT‡¶, BERNARD MOSS‡, AND HILARY KOPROWSKI*

*Wistar Institute of Anatomy and Biology, 36th Street at Spruce, Philadelphia, PA 19104; †Transgene S.A., 11 Rue de Molsheim, 67000 Strasbourg, France; and ‡Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, MD 20205

Contributed by Hilary Koprowski, July 30, 1984

ABSTRACT **Inoculation of rabbits and mice with a vaccinia-rabies glycoprotein recombinant (V-RG) virus resulted in rapid induction of high concentrations of rabies virus-neutralizing antibodies and protection from severe intracerebral challenge with several strains of rabies virus. Protection from virus challenge also was achieved against the rabies-related Duvenhage virus but not against the Mokola virus. Effective immunization by V-RG depended on the expression of a rabies glycoprotein that registered proline rather than leucine as the eighth amino acid from its NH_2 terminus (V-RGpro8). A minimum dose required for effective immunization of mice was 10^4 plaque-forming units of V-RGpro8 virus. β-propiolactone-inactivated preparations of V-RGpro8 virus also induced high levels of rabies virus-neutralizing antibody and protected mice against intracerebral challenge with street rabies virus. V-RGpro8 virus was highly effective in priming mice to generate a secondary rabies virus-specific cytotoxic T-lymphocyte response following culture of lymphocytes with either ERA or PM strains of rabies virus.**

Rabies is a disease of the central nervous system of major importance to human and veterinary medicine. The etiologic agent, rabies virus, is composed of five structural proteins and a linear, single-stranded RNA genome of negative sense. The rabies virus glycoprotein (G) forms surface projections through the viral lipid envelope and is the only protein capable of inducing and reacting with virus-neutralizing antibody (VNA) (1, 2). Several studies have established that the isolated G is capable of protecting animals against rabies (for review, see ref. 3).

Recently, cloned cDNA copies of the G mRNA from two rabies virus strains have been isolated and sequenced (4, 5). Expression of either G in bacterial hosts has so far failed to yield a product capable of immunizing animals against rabies (5–7). In order to provide post-translational modifications potentially necessary for production of authentic rabies virus G, a vector system allowing expression of rabies G in eukaryotic hosts was sought. To this end, successful expression, immunization, and protection has been reported with infectious vaccinia virus recombinants containing foreign genes such as hepatits B surface antigen, influenza virus hemagglutinin, and herpes simplex virus glycoprotein D (8–12).

This study compares the biologic and protective properties of two vaccinia virus recombinants expressing ERA strain rabies G proteins differing at a single amino acid residue. We report that infection of cells with either vaccinia virus recombinant resulted in the expression of a novel rabies G; however, only one of these products induced virus-neutralizing activity, cytotoxic T-cell memory, and protection against an intracerebral challenge with rabies virus.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Cells and Viruses. Monolayer cultures of BHK-21 clone 13 cells (13) and NA neuroblastoma cells of A/J mouse origin (14) were grown at 37°C in Eagle's minimum essential medium supplemented with 10% fetal calf serum as described (15). The ERA strain of rabies virus (16) was propagated in BHK-21 cells. The PM strain of rabies virus, grown in Vero cells and inactivated by β-propiolactone (βPL), was a gift from the Institute Merieux (Bio Vero Lot S-1163). Challenge viruses included the MD5951 strain of street rabies virus (17), obtained from G. M. Baer of the Centers for Disease Control (Atlanta, GA); a human isolate (HI5) street rabies virus; and rabies-related Duvenhage virus (18) and Mokola strain IbAn 27377 (19) virus. A stock of each challenge virus was prepared from NA or BHK-21 cells and titrated by intracerebral inoculation into 5- to 6-wk-old ICR mice. Additional street rabies viruses used for testing the virus-neutralizing activity of antisera were isolated in 1983 from *Eptesicus fuscus* bat in Ontario, Canada, in 1974 from salivary glands of a red fox in France, and in 1956 from human brain in China (strain CTN-1) provided through the World Health Organization (Geneva); also used was rabies-related Lagos bat virus (19). Wild-type vaccinia viruses (strains WR and Copenhagen) were prepared in tissue culture and purified from cytoplasmic extracts by sucrose gradient centrifugation (20). Vaccinia recombinant viruses containing cloned rabies G cDNA were constructed by using methods previously described (21–23). The vaccinia–rabies glycoprotein recombinant (V-RG) virus containing the coding sequence for proline as the eighth amino acid of the rabies virion G is designated V-RGpro8 (VVTGgRAB in ref. 23), and that which codes for leucine as the eighth amino acid is designated V-RGleu8. Infectivity titers of wild-type vaccinia and V-RG recombinant viruses were determined by a plaque assay on CER cells as described for rabies virus (24).

Preparation of Inactivated V-RG Vaccines. BHK-21 cells at 80–90% confluence were infected with vaccinia (Copenhagen) or V-RGpro8 virus at an input multiplicity of 0.1 plaque-forming units (pfu) per cell. After a 1 hr adsorption at room

Abbreviations: V-RG, vaccinia-rabies glycoprotein recombinant; V-RGpro8, V-RG expressing proline at position 8; G, rabies virus glycoprotein; VNA, virus-neutralizing antibodies; βPL, β-propiolactone; V-RGleu8, V-RG expressing leucine at position 8; pfu, plaque-forming units; CTL, cytotoxic T lymphocyte(s); LU30, 30% lytic units.

§Present address: ARC-Animal Breeding Research Organization, King's Buildings, West Mains Road, Edinburgh, EH9 3JQ, U.K.
¶Present address: Paterson Laboratories, Christie Hospital and Holt Radium Institute, Manchester, M20 9BX, U.K.

temperature, cells were cultured at 37°C with Eagle's minimum essential medium supplemented with 0.2% bovine serum albumin until the cytopathic effect reached 50–75%. Cells were scraped from culture vessels, pelleted, washed once with phosphate-buffered saline (pH 7.4), and swelled for 15 min at 0°C in 10 mM Tris·HCl, pH 7.6/10 mM KCl/1.5 mM $MgCl_2$/2 mM phenylmethylsulfonyl fluoride. Cells were homogenized twice in a Dounce homogenizer, and the nuclei were pelleted. The supernatant represented a crude cell extract of vaccinia or V-RGpro8 viruses. A portion of this extract was inactivated by βPL (1:4000) as described elsewhere (25). The absence of live virus in these preparations was confirmed by infecting monolayers of BHK-21 cells and observing for virus-induced cytopathic effect. A blind passage of culture fluid from these cells onto fresh cultures of BHK-21 cells was performed 5 days after infection. No infectious virus could be detected. Part of the V-RGpro8 virus-infected cell extract was then used for isolation and purification of recombinant virus by sucrose gradient centrifugation and treated with βPL. The remaining extract was adjusted to 2% Triton X-100 and centrifuged for 1 hr at 23°C at 107,000 × *g*. The solubilized G was isolated from the supernatant by adsorption to an affinity column prepared with an anti-rabies virus-G monoclonal antibody (26). The eluted G was treated with βPL. Protein concentrations were determined with bovine serum albumin as the standard (27).

Animals. Female 5- to 6-wk-old ICR mice (Dominion Laboratories, Dublin, VA) and 5- to 8-wk-old A/J mice (The Jackson Laboratory) were used in these experiments. New Zealand White female rabbits were purchased from Hazleton Dutchland (Aberdeen, MD).

Immunization and Challenge Protocols. Rabbits were inoculated by intradermal injection of 2×10^8 pfu of V-RG virus distributed into three separate sites on the back. ICR mice were infected intradermally by scarification of tail skin or by injection into the footpad with either wild-type or recombinant vaccinia viruses (10^9 pfu/ml). When βPL-inactivated virus was used, mice were inoculated with two intraperitoneal injections (0.5 ml) 7 days apart. Immunized mice and rabbits were challenged with street rabies virus by intracerebral inoculation with 2400 and 24,000 mouse LD_{50}, respectively, and were observed for a minimum period of 3 mo.

Antibody Titrations. Rabies VNA titers were measured by a modified rapid fluorescent focus inhibition technique (28) against ERA strain rabies virus. Titers are expressed as the highest serum dilution that was capable of reducing the number of rabies virus-infected cells by 50%. A neutralization index was determined by comparing the number of infected cells in control cultures with the number of infected cells in cultures incubated in the presence of antibody-containing serum and expressed as the $\log_{10}$ virus reduction per ml of undiluted serum (29). The virus neutralization titers for antibodies directed against vaccinia virus was determined by a plaque reduction assay with monolayers of CER cells (15).

Detection of Antigen by Immunofluorescence. Rabies G in V-RG virus-infected cells was visualized by indirect immunofluorescence in live or acetone-fixed cells using anti-G antiserum as described elsewhere (29).

Cytotoxic T-Lymphocyte (CTL) Responses. A/J mice were inoculated intraperitoneally with 10^7 pfu of ERA rabies virus or intravenously with 10^5 pfu of wild-type vaccinia or V-RGpro8 virus. Primary CTL responses were assayed at 6 days after infection (30), and secondary *in vitro* CTL responses at 4 wk after infection. To generate secondary CTL, spleen cells from primed mice were cultured at 2.5×10^6 cells per ml with dilutions of βPL-inactivated viruses in medium (31) containing 10% fetal calf serum. After incubation for 5 days at 37°C in 5% CO_2/95% air, the cells were washed in Eagle's minimum essential medium with 2% fetal calf serum and titrated for cytotoxicity in a 6-hr ^{51}Cr-release assay as described (30). Infection of NA target cells with wild-type vaccinia or V-RGpro8 viruses was carried out as described for rabies virus (30) except that the infected cells were incubated in siliconized Petri dishes for 5–6 hr to allow expression of surface antigens. Cells were then labeled with ^{51}Cr and used as targets. Results are presented as 30% lytic units (LU30) and take into account the spontaneous release of ^{51}Cr into the medium (10–24%) and the maximum release in detergent. One LU30 is defined as the number of effector cells required to achieve 30% specific ^{51}Cr release. A large number of LU30 per culture indicates a potent CTL population.

RESULTS

Expression of Rabies G in Vaccinia Virus Vectors. The rabies-specific G cDNA isolated by Anilionis *et al.* (4) was inserted into the *Bam*HI site of plasmid pGS20 so as to be controlled by an early vaccinia virus promoter translocated within the thymidine kinase gene (22). The chimeric gene formed in this manner contains the vaccinia RNA start site juxtaposed with the rabies translational initiation codon so as to avoid the production of a fusion protein. This plasmid construct was used to transfect vaccinia virus (strain WR)-infected cells to prepare a recombinant virus that contained the rabies G cDNA inserted into the thymidine kinase locus (V-RGleu8). Successful expression of a novel rabies G in V-RGleu8 virus-infected BHK-21 cells resulted in a protein that was metabolically labeled with [^{35}S]methionine and [^3H]glucosamine, was immunoprecipitable with polyclonal rabbit anti-G antibodies, but which migrated faster than rabies virion G in $NaDodSO_4$/polyacrylamide gel. In V-RGleu8 virus-infected BHK-21 cells, the pattern of immunofluorescence suggested that the protein expressed by V-RGleu8 virus was not in a native configuration (Fig. 1). First, the fluorescence that is characteristic of the rabies virus G on the surface of cells was weak in V-RGleu8 virus-infected cells, where the majority of antigen was detected within the cytoplasm (Fig. 1 *A* and *B*). Secondly, a panel of anti-G monoclonal antibodies that bind only to native rabies virus G failed to detect the V-RGleu8 virus-expressed antigen (not shown). Moreover, injection of V-RGleu8 virus into animals failed to induce rabies VNA (Table 1) and to protect against rabies.

Amino acid analysis of the NH_2 terminus of the rabies virus G (32) revealed a discrepancy at amino acid position 8 (proline) with the predicted sequence (leucine) of the original cDNA clone (4). By sequencing this entire viral G gene, this amino acid change and one other at position 399 (leucine to valine) were identified (ref. 33; data not shown). Assuming that the change near the NH_2 terminus might have a greater impact on the structure formation of nascent G, we modified the cDNA clone by site-directed mutagenesis to rectify the amino acid at position 8 (23). In addition, the guanosine tail originally introduced for cloning the cDNA was removed since it may impede expression (23). This modified DNA was inserted into plasmid pTG186 (23) and subsequently transferred into vaccinia virus to generate the recombinant designated V-RGpro8. The Copenhagen strain of vaccinia virus used for human vaccination was used as the vector. Infection of BHK-21 cells by V-RGpro8 virus resulted in expression of a rabies G on the cell surface and in cytoplasm detected by immunofluorescence (Fig. 1 *C* and *D*). The protein expressed by this recombinant virus reacted with a panel of anti-G monoclonal antibodies in a pattern identical with native rabies virus G (23).

Induction of VNA and Protection Against Rabies. Inoculation of rabbits and mice with V-RGpro8 virus resulted in a rapid induction of rabies VNA (Table 1). In rabbits, rabies

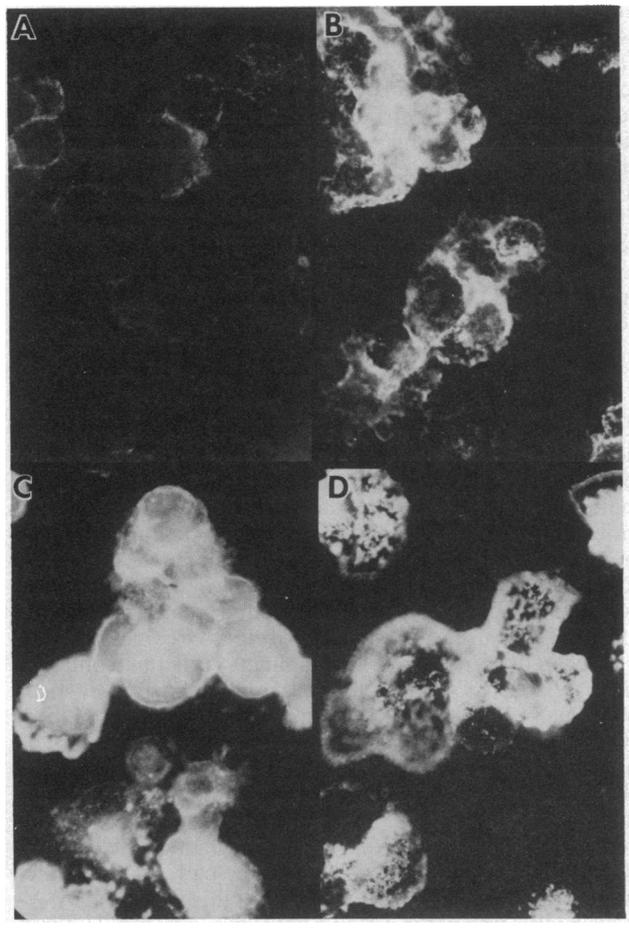

FIG. 1. Detection of rabies G antigen in V-RG virus-infected cells. Monolayers of BHK-21 cells were infected with 0.1 pfu per cell of virus and cultured for 16 hr. Antigen was visualized by indirect immunofluorescence using rabbit antirabies virus G antiserum unfixed (*A* and *C*) or acetone-fixed (*B* and *D*) cells infected with V-RGleu8 (*A* and *B*) or V-RGpro8 (*C* and *D*) viruses.

VNA titers at 5, 11, and 14 days after inoculation were 800, 10,000, and >30,000, respectively. Vaccinia VNA titers after 14 days were substantially lower. Rabbit serum (day 14) neutralized between $10^{5.3}$ and $10^{6.6}$ tissue culture ID_{50} of ERA rabies virus, and three street rabies virus isolates previously shown to differ from the ERA strain in their reactivity with a panel of anti-rabies virus G monoclonal antibodies. Neutralization indices against rabies-related Duvenhage, Lagos bat, and Mokola viruses were $10^{6.2}$, $10^{3.1}$, and $10^{3.4}$, respectively. These results, which are comparable to those obtained with anti-ERA rabies virus antiserum, demonstrate that Duvenhage virus is more closely related to rabies than are rabies-related Lagos bat and Mokola viruses.

Three of four rabbits vaccinated with V-RGpro8 virus resisted a severe intracerebral challenge with 24,000 mouse LD_{50} of MD5951 rabies virus, whereas all five unvaccinated control rabbits died from rabies after 12–15 days (Table 1). The one vaccinated rabbit that died from rabies survived until 21 days after challenge.

Inoculation of mice with V-RGpro8 virus, by either scarification or injection into the footpad, resulted in rabies VNA titers of 30,000 or higher after 14 days. All mice were protected against challenge with either HI5 or MD5951 rabies viruses or with the rabies-related Duvenhage virus. No protection was seen after challenge with Mokola virus. Mice inoculated with wild-type vaccinia or V-RGleu8 virus did not develop rabies VNA and were not protected against rabies.

A minimum dose of V-RGpro8 virus capable of protecting 50% of recipient mice was 10^4 pfu (Fig. 2). In this experiment, mice were inoculated in the footpad and challenged intracerebrally with 2400 mouse LD_{50} of MD5951 rabies virus after 15 days. Levels of rabies VNA were determined at 7 and 14 days (Fig. 2).

Cellular Immune Response Induced by V-RGpro8 Virus. Rabies viruses induce a strong rabies-specific primary CTL response in A/J mice (31); in contrast, CTL induced by V-RGpro8 virus were predominantly directed against vaccinia virus-infected target cells (not shown). However, inoculation with V-RGpro8 virus effectively primed mice for a secondary CTL response after culture of lymphocytes with βPL-inactivated ERA or PM rabies viruses (Fig. 3 *A* and *B*). These CTL were specific only for target cells expressing rabies G (i.e., infected with ERA rabies virus or with V-RGpro8 virus). Target cells infected with V-RGpro8 virus were comparatively resistant to lysis, perhaps reflecting differences in density or display of rabies G. In contrast, lymphocytes from V-RGpro8-primed mice lysed only vaccinia or V-RGpro8 virus-infected cells after stimulation with inactivated, purified V-RGpro8 virus (Fig. 3*C*). Spleen cells from mice primed with vaccinia virus generated no CTL activity after stimulation with rabies viruses. In another experiment, lymphocytes from mice primed with ERA rabies virus generated a strong secondary CTL response after culture with either inactivated PM or ERA rabies virus (Fig. 3 *D* and *E*), whereas inactivated V-RGpro8 virus was ineffective at the dilutions tested (Fig. 3*F*) despite evidence that this preparation contained rabies G (see below).

Immunogenicity of Inactivated V-RGpro8 Virus. The abili-

Table 1. Induction of VNA and protection from rabies by vaccinia recombinant viruses

Animals/ inoculation route	Vaccine*	VNA titers: Rabies Day 0	Rabies Day 5	Rabies Day 11	Rabies Day 14	Vaccinia Day 14	Protection†
Rabbits/ intradermal	V-RGpro8	<10	800	10,000	>30,000	250	3/4
	V-RGleu8	<10	—	—	<10	—	—
	None	<10	—	—	<10	—	0/5
Mice/ intradermal	V-RGpro8	<10	—	—	>30,000	250	12/12
	V-RGleu8	<10	—	—	<10	—	0/12
	Vaccinia	<10	—	—	<10	250	0/12
Mice/ footpad	V-RGpro8	<10	—	—	>30,000	1250	12/12
	V-RGleu8	<10	—	—	<10	—	0/12
	Vaccinia	<10	—	—	<10	1250	0/12

*Vaccine was inoculated on day 0 using 2×10^8 pfu (intradermal) or 5×10^7 pfu (footpad) of virus.
†A challenge dose of 2400 or 24,000 mouse LD_{50} of MD5951 rabies virus was given on day 14 to mice and rabbits, respectively, by intracerebral inoculation.

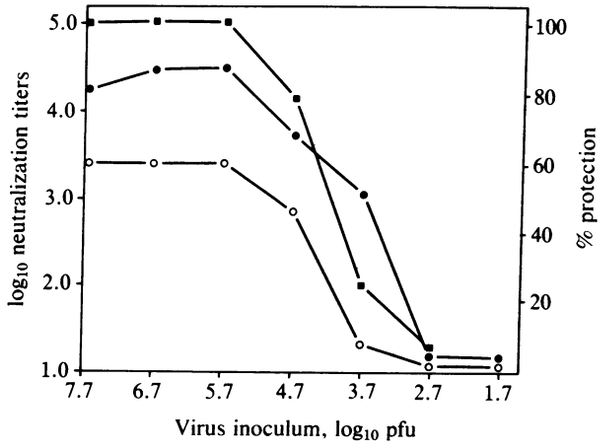

FIG. 2. Minimum protective dose of V-RGpro8 virus. Groups of 10 mice were inoculated in the footpad with serial 10-fold dilutions of V-RGpro8 virus. Levels of rabies VNA were determined 7 (○) and 14 (●) days after infection. On day 15, mice were challenged intracerebrally with 2400 mouse LD_{50} of MD5951 rabies virus (■).

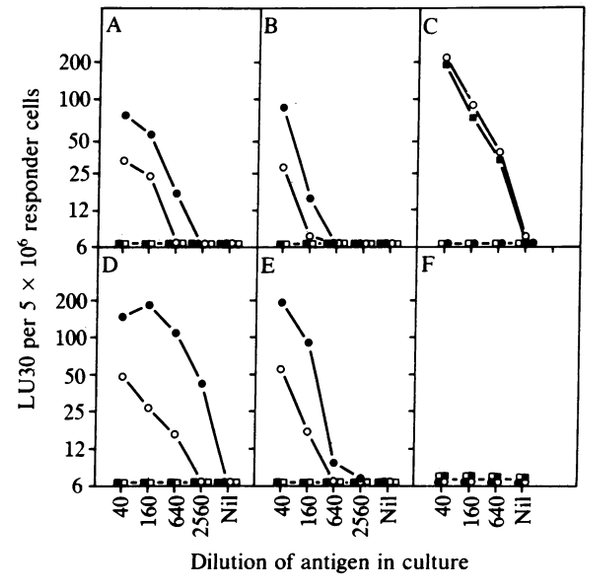

FIG. 3. Secondary CTL response stimulated by V-RGpro8 and rabies viruses *in vitro*. A/J mice were inoculated intravenously with 10^5 pfu of V-RGpro8 virus (*A*, *B*, and *C*) or intraperitoneally with 10^7 pfu of ERA rabies virus (*D*, *E*, and *F*). Four weeks later, spleen cells were cultured with dilutions of βPL-inactivated ERA (*B* and *E*) or PM (*A* and *D*) rabies virus or with βPL-inactivated V-RGpro8 virus (*C* and *F*). After 5 days, each culture was titrated for CTL activity, which is expressed as LU30 generated per 5 × 10^6 responder spleen cells. Target cells: uninfected (□), ERA rabies virus-infected (●), vaccinia virus-infected (■), or V-RGpro8 virus-infected (○) ^{51}Cr-labeled NA cells. Specific lysis due to anti-rabies antibody plus complement was 0%, 95.1%, 0%, and 93.2%, respectively.

ty of βPL-inactivated preparations of V-RGpro8 virus to induce an anti-rabies immune response was tested. Extracts of V-RGpro8 virus-infected cells, purified V-RGpro8 virus, and G isolated from V-RGpro8 virus-infected cell extracts by using an affinity column prepared with anti-rabies virus G antibody, were inactivated and inoculated intraperitoneally into mice. The mice were inoculated again after 7 days and challenged intracerebrally with 240 LD_{50} of MD5951 rabies virus after a further 7 days. All three preparations induced high levels of rabies VNA and protected against rabies (Table 2).

DISCUSSION

The construction of vaccinia virus recombinants expressing genes derived from pathogenic agents has great potential for the production of vaccines. In this report we demonstrate the effectiveness of live and inactivated experimental rabies vaccines prepared by this technology. Initially, we constructed a WR strain vaccinia recombinant virus incorporating the ERA rabies virus G cDNA sequence described by Anilionis *et al.* (4), which codes for leucine at position 8 of the rabies virion G. However, direct amino acid sequencing of rabies virus G established that the eighth residue was proline. The difference in nucleotide sequence between the original cDNA clone and viron RNA, resulting in this amino acid substitution, could have arisen during the cloning procedure or in the transcription of virion RNA to mRNA. In any case, since we could not be sure whether the original cDNA coded for a functional gene product, the cloned sequence was changed by site-directed mutagenesis to code for proline at position 8 and inserted into a second vaccinia vector derived from the Copenhagen vaccinia strain (23). Both recombinant viruses (V-RGleu8 and V-RGpro8) produced a protein of similar size that was detected in fixed preparations of infected cells by immunofluorescence using monospecific antiserum raised against rabies virus G. However, the V-RGpro8 virus-expressed antigen, but not the V-RGleu8 antigen, was detectable at high density on the surface of infected cells and reacted with monoclonal antibodies recognizing native rabies virus G. This information indicates that the V-RGleu8 virus rabies G has an altered antigenic structure. Remarkably, a single amino acid substitution near the NH_2 terminus evidently results in a generally altered conformation of G, which may affect either post-translational modification or

Table 2. Induction of VNA and protection from rabies by inactivated preparations from V-RGpro8 recombinant virus

Vaccine*	Titer before inactivation, $\log_{10}$ pfu/ml	Protein concentration, μg per mouse†	Rabies VNA titers		
			Day 7	Day 14	Protection‡
V-RGpro8 virus-infected cell extract	7.5	140	80	8000	12/12
V-RGpro8 purified virus	8.6	9	270	4000	12/12
V-RGpro8 purified rabies G	<1.0§	50	120	15000	12/12
Vaccinia virus-infected cell extract	8.6	900	<10	<10	0/12
Unvaccinated controls	—	—	—	<10	0/12

*Vaccines were prepared from infected BHK-21 cells as described in *Materials and Methods* and inactivated with βPL.
†Total protein in two intraperitoneal inoculations given on days 0 and 7.
‡A challenge dose of 240 mouse LD_{50} of MD5951 rabies virus was given on day 14 to mice by intracerebral inoculation.
§No infectivity detected in undiluted sample.

transport. The rabies G (Leu-8) was also defective when expressed in the Copenhagen vaccinia virus vector (not shown).

Inoculation of mice and rabbits with V-RGpro8 virus induced high levels of rabies VNA. The titers obtained with a single inoculation of this recombinant vaccinia virus were consistently higher than those seen after repeated immunization with inactivated rabies viral vaccines of the type currently used for vaccination (1, 33). V-RGpro8 virus effectively protected animals from rabies. Mice and rabbits survived intracerebral challenge with 2400 and 24,000 mouse LD_{50} of street rabies virus, respectively. This can be regarded as a severe test of immunity. These results of pre-exposure immunization experiments indicate that V-RGpro8 virus has potential as a vaccine for human and/or veterinary use.

In humans, rabies vaccination is used primarily for treatment after exposure to the virus. It has been postulated that not only VNA but also CTL responses are important in post-exposure protection (34, 35). Mice immunized with V-RGpro8 virus generated a substantial secondary cytotoxic response *in vitro* after re-exposure of lymphocytes to PM or ERA rabies viruses (Fig. 3) or *in vivo* after inoculation of V-RGpro8 virus-immunized mice with ERA rabies virus (unpublished data). The CTL generated were specific for rabies G and lysed target cells infected with V-RGpro8 or ERA rabies viruses. A similar priming effect also has been demonstrated after immunization with a vaccinia recombinant virus expressing the influenza virus hemagglutinin (36).

Despite the ability of V-RGpro8 virus to induce CTL memory specific for rabies G, primary rabies-specific CTL responses were weak. Since V-RGpro8 did induce a primary vaccinia-specific CTL response, this finding may reflect some form of immunodominance; however, the mechanisms involved are unclear (37).

Live vaccinia virus has a long history of safe use as a vaccine for humans, despite a low incidence of serious complications (38). Reintroduction of vaccinia virus-based vaccines may be controversial; therefore, we have evaluated the immunogenicity of purified inactivated V-RGpro8 virus and the rabies G isolated from V-RGpro8 virus-infected cells. Both preparations induced rabies VNA and protected mice against rabies. Induction of VNA by inactivated V-RGpro8 virus implies that the rabies G is closely associated with the V-RGpro8 virion. Immunoelectron microscopy should clarify whether the rabies G is a component of the viral membrane. However, these initial results suggest the possibility that inactivated V-RGpro8 virus could also be used as a vaccine against rabies.

This work was supported by Research Grants AI-09706 and AI-18883 from the National Institute of Allergy and Infectious Diseases.

1. Wiktor, T. J., Gyorgy, E., Schlumberger, H. D., Sokol, F. & Koprowski, H. (1973) *J. Immunol.* **110,** 269–276.
2. Cox, J. H., Dietzschold, B. & Schneider, L. G. (1977) *Infect. Immun.* **16,** 754–759.
3. Wunner, W. H., Dietzschold, B., Curtis, P. J. & Wiktor, T. J. (1983) *J. Gen. Virol.* **64,** 1649–1656.
4. Anilionis, A., Wunner, W. H. & Curtis, P. J. (1981) *Nature (London)* **294,** 275–278.
5. Yelverton, E., Norton, S., Obijeski, J. F. & Goeddel, D. V. (1983) *Science* **219,** 614–620.
6. Lathe, R. F., Kieny, M. P., Schmitt, D., Curtis, P. & Lecocq, J. P. (1984) *J. Mol. Appl. Genet.* **2,** 331–342.
7. Malek, L. T., Soostmeyer, G., Garvin, R. T. & James, E. (1984) in *Modern Approaches to Vaccines: Molecular and Chemical Basis of Virus Virulence and Immunogenicity*, eds. Channock, R. M. & Lerner, R. A. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), Vol. 1, pp. 203–208.
8. Smith, G. L., Mackett, M. & Moss, B. (1983) *Nature (London)* **302,** 490–495.
9. Smith, G. L., Murphy, B. R. & Moss, B. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 7155–7159.
10. Moss, B., Smith, G. L., Gerin, J. L. & Purcell, R. H. (1984) *Nature (London)* **311,** 67–69.
11. Panicali, D., Davis, S. W., Weinberg, R. L. & Paoletti, E. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 5364–5368.
12. Paoletti, E., Lipinskas, B. R., Samsonoff, C., Mercer, S. & Panicali, D. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 193–197.
13. Stoker, M. & MacPherson, I. (1964) *Nature (London)* **203,** 1355–1357.
14. Clark, H. F. (1980) *Infect. Immun.* **27,** 1012–1022.
15. Wiktor, T. J. (1973) in *Laboratory Techniques in Rabies*, World Health Organization Monograph No. 23, eds. Kaplan, M. & Koprowski, H. (World Health Organization, Geneva), pp. 101–123.
16. Clark, H. F. & Wiktor, T. J. (1972) in *Strains of Human Viruses*, ed. Plotkin, S. A. (Karger, Basel, Switzerland), pp. 177–182.
17. Smith, J. S., McClelland, C. L., Reid, F. L. & Baer, G. M. (1982) *Infect. Immun.* **35,** 213–221.
18. Meredith, C. D., Rossouw, A. P. & Van Praag Koch, H. (1971) *S. Afr. Med. J.* **45,** 767–769.
19. Shope, R. E., Murphy, F. A., Harrison, A. K., Causey, O. R., Kemp, G. E., Simpson, D. I. H. & Moore, D. L. (1970) *J. Virol.* **6,** 690–692.
20. Joklik, W. K. (1962) *Virology* **18,** 9–18.
21. Moss, B., Smith, G. L. & Mackett, M. (1983) in *Gene Amplification and Analysis*, eds. Papas, T. S., Rosenberg, M. & Chirikjian, J. K. (Elsevier/North-Holland, New York), Vol. 3, pp. 201–213.
22. Mackett, M., Smith, G. L. & Moss, B. (1984) *J. Virol.* **49,** 857–864.
23. Kieny, M. P., Lathe, R., Drillien, R., Spehner, D., Skory, S., Schmitt, D., Wiktor, T., Koprowski, H. & Lecocq, J.-P., *Nature (London)*, in press.
24. Lafon, M., Wiktor, T. J. & Macfarlan, R. I. (1983) *J. Gen. Virol.* **64,** 843–851.
25. Wiktor, T. J., Aaslestad, H. G. & Kaplan, M. M. (1972) *Appl. Microbiol.* **23,** 914–918.
26. Dietzschold, B., Wiktor, T. J., Wunner, W. H. & Varrichio, A. (1983) *Virology* **124,** 330–337.
27. Bramhall, S., Noack, N., Wu, M. & Loewenberg, J. R. (1969) *Anal. Biochem.* **31,** 146–148.
28. Reagan, K. J., Wunner, W. H., Wiktor, T. J. & Koprowski, H. (1983) *J. Virol.* **48,** 660–666.
29. Wiktor, T. J. & Koprowski, H. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 3938–3942.
30. Macfarlan, R. I., Dietzschold, B., Wiktor, T. J., Kiel, M., Houghten, R., Lerner, R. A., Sutcliffe, J. G. & Koprowski, H. (1984) *J. Immunol.*, in press.
31. Wiktor, T. J., Doherty, P. C. & Koprowski, H. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 334–338.
32. Dietzschold, B., Wiktor, T. J., Macfarlan, R. I. & Varrichio, A. (1982) *J. Virol.* **44,** 595–602.
33. Wunner, W. H., Smith, C. L., Lafon, M., Ideler, J. & Wiktor, T. J. (1983) in *Nonsegmented Negative Strand Viruses*, eds. Bishop, D. H. L. & Compans, R. W. (Academic, San Diego, CA), pp. 279–284.
34. Plotkin, S. A., Wiktor, T. J., Koprowski, H., Rosenoff, E. I. & Tint, H. (1976) *Am. J. Epidemiol.* **103,** 75–80.
35. Wiktor, T. J. (1978) *Dev. Biol. Stand.* **40,** 255–264.
36. Bennink, J. R., Yewdell, J. W., Smith, G. L., Moller, C. & Moss, B. (1984) *Nature (London)*, in press.
37. Wybier-Franqui, J., Gomard, E. & Levy, J. P. (1982) *Cell. Immunol.* **68,** 287–301.
38. Lane, J. M., Ruben, F. L., Neff, J. M. & Millar, J. D. (1970) *J. Infect. Dis.* **122,** 303–309.