ANTIGENIC VARIANTS OF RABIES VIRUS*

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Rabies viruses isolated from different animal species in various parts of the world were in the past considered to be antigenically closely related. Only when the antibodies produced in animals immunized with whole virions or viral components were assayed by the plaque reduction method, were some minor differences detected in the antigenic composition of various rabies strains (1). On the other hand, monoclonal antibodies, produced by the hybrids of mouse myeloma cells with splenocytes of rabies-immunized BALB/c mice and directed against either nucleocapsid or glycoprotein of the virus, provided categorical evidence of antigenic differences among several fixed and street rabies viruses (2).

The notion of antigenic differences among rabies virus strains may be of particular importance in rabies prophylaxis. Most vaccines used in humans for protection after exposure are derived from a rabies virus originally isolated and adapted to rabbit brain by Pasteur in 1882 (3). It was assumed that this strain had sufficient cross-reactivity with all field strains of rabies virus in different geographical areas to protect exposed individuals.

Unfortunately, the postexposure treatment of rabies is not 100% effective. Failure to protect exposed individuals has been attributed either to low potency of the vaccine or to a delay in beginning treatment. Occasionally, however, failure occurs even when the vaccine can be assumed to be potent and the treatment prompt (4, 5). Antigenic differences among strains of rabies virus were suspected to be responsible, but until now, there was no way to detect these differences.

The purpose of the studies presented in this paper was: (*a*) either to produce variants of rabies virus in the laboratory or to select variants from naturally occurring (street) viruses, (*b*) to study antigenic specificities of these variants by means of monoclonal antibodies, and (*c*) to undertake cross-protection tests in mice immunized with either standard rabies vaccines or vaccines produced by variants and challenged with homologous and variant viruses.

Materials and Methods

Hybridoma Monoclonal Antibodies. Hybridomas, which secrete monoclonal antibodies specific for rabies virus nucleoproteins, glycoproteins, and two membrane proteins, were produced by the fusion of P3 × 63 Ag8 cells with splenocytes of BALB/c mice immunized with several strains of rabies virus (2, 6). Mice were inoculated twice at 4- to 8-wk intervals with an inactivated virus suspension. 3–4 d after the second injection, mouse splenocytes were fused with mouse myeloma cells and incubated in selective hypoxanthine-aminopterin-thymidine

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(HAT)[1] medium. The selection and cloning of hybridomas produced by the fusion of the two types of cells have been described elsewhere (2). In the present investigation, 21 hybridomas specific for nucleocapsid (7) and 24 hybridomas specific for glycoprotein (8) of rabies virus were used.

Viruses

LABORATORY STRAINS. *CVS-11*, the challenge standard virus (CVS) (9) was propagated in baby hamster kidney (BHK-21) cells as previously described (10). A virus pool representing second passage after two serial clonings had an infectivity titer of 6×10^7 plaque-forming units (PFU)/ml; *CVS-DEV*, the vaccine strain (11), received from Eli Lilly and Co., Indianapolis, Ind., was propagated for three passages in BHK-21 cells; *PV-11*, the vaccine strain, received from Bureau of Biologics, Bethedsa, Md., was propagated for 58 passages in human diploid cell cultures (12).

FIELD ISOLATES OF HUMAN ORIGIN. Eight coded rabies virus field isolates were provided by Dr. G. M. Baer, Center for Disease Control (CDC), Atlanta, Ga. The original material was homogenized and clarified by centrifugation. The supernatant fluid was injected intracerebrally into groups of 5-wk-old ICR mice. When paralyzed, animals were sacrificed, and their brains were harvested for use in nucleocapsid antigen analysis as previously described (6), and for challenge experiments (see below).

BHK-21 cells in culture were infected with the clarified original brain suspensions, and virus was propagated in those cells for two or three additional passages. When the tissue culture fluids from infected cells contained at least 1,000 infective units of virus/ml, they were used in the neutralization assay.

Virus Neutralization. The susceptibility of CVS-11, CVS-11-derived variants, and street rabies viruses to neutralization by different hybridoma antibodies was evaluated by determining the virus neutralization index (7). Briefly, serial dilutions of virus were mixed with constant amounts of hybridoma medium and incubated at 37°C for 1 h. In the case of CVS-11 and CVS-11 variants, the remaining infectivity was determined by infecting CER hamster cells' monolayers (13) and counting plaques produced after 4 d of incubation under nutrient agarose overlay. In the case of street viruses, the remaining infectivity was determined by infection of BHK-21 cells and staining foci of infected cells by fluorescent antibody (FA) staining technique. The neutralization index was determined by substracting either the number of plaques or FA-foci/ml in cultures infected with virus and antibody mixtures, from the number of plaques or FA-foci/ml in mixtures of virus and normal medium.

Titration of Neutralizing Antibody in Serum of Immunized Mice. The level of virus-neutralizing antibody in immunized mice was determined by the rapid fluorescent focus-inhibition technique that has been described elsewhere (13).

Immunofluorescent Antibody Staining for Nucleocapsid Antigen. The reactivity of anti-nucleocapsid monoclonal antibodies with antigen present in brains of rabies virus-infected mice was detected by the indirect FA staining procedure as previously described (6).

Selection of Variants. Serial 10-fold dilutions of virus were prepared and mixed with equal volumes of Eagle's minimal essential medium (MEM) or hybridoma antibody diluted in MEM. After 1 h of incubation at 37°C, 0.1 ml of virus antibody mixture was added to CER cell monolayers (14) grown in 60-mm Petri dishes. After adsorption for 1 h, 5 ml of nutrient agarose, with or without hybridoma antibody, was poured over the cell layers. Plates were incubated at 35°C for 4 d and were scored for the presence of rabies virus-induced plaques, which were visible without staining. At the end point of neutralization, well separated plaques were collected with a Pasteur pipette and dispersed in 5 ml MEM containing 1×10^6 freshly trypsinized BHK-21 cells in T-25 Falcon plastic tissue culture flasks (Falcon Labware, Div. of Becton, Dickinson, & Co., Oxnard, Calif.). Portions of 0.5 ml infected cells were transferred into wells of four-chamber Lab-Tek tissue culture slides (Lab-Tek Products, Div. Miles Laboratories, Inc., Naperville, Ill.). The T-25 flasks and Lab-Tek slides were incubated for 3 d.

[1] *Abbreviations used in this paper*: BHK, baby hamster kidney; CVS, challenge standard virus; FA, fluorescent antibody staining; HAb, hybridoma antibody(ies); HAT, hypoxanthine-aminopterin-thymidine medium; HRIG, human rabies immune globulin; MEM, Eagle's minimal essential medium; PFU, plaque-forming units.

The presence of virus in the culture was detected by FA staining.

Several clones of virus obtained by this technique from each neutralization test were assayed for resistance to neutralization by the hybridoma antibody used for their selection.

Unless otherwise stated, 1:5 dilution of hybridoma antibody was used to select variants and to test resistance to neutralization.

Vaccine. Concentrated, β-propiolactone-inactivated vaccine was prepared as previously described (15) from BHK-21 cells infected with two fixed viruses, CVS-11 and PV-11, and with selected variant strains.

Immunization and Challenge. 6-wk-old ICR mice were injected intraperitoneally with 0.5 ml of a 1:50 dilution of vaccine twice weekly. Animals were challenged 7 d after the last dose of vaccine by intracerebral inoculation with serial 10-fold dilutions of homologous and heterologous viruses. The infectivity titers of challenge virus were determined by intracerebral inoculation into unvaccinated control mice. Animals were examined daily for 3 wk for signs of rabies. Some animals were sacrificed when *in extremis*, and the presence of rabies virus was confirmed by FA staining of brain tissue impressions and by reisolation of virus in BHK-21 cell cultures, or by intracerebral inoculation into mice.

Results

Selection of Variants. An example of selection of a variant virus by exposing the parental CVS to an overneutralizing amount of monoclonal antiglycoprotein antibody is shown in Table I. It can be seen that in cultures containing hybridoma antibody 101-1 diluted 1:20, the infectivity titer of the CVS decreased from $10^{7.7}$ to $10^{3.7}$ PFU/ml. The same antibody diluted 1:100 decreased the titer to $10^{5.2}$ PFU/ml. Virus grown from 21 individual plaques obtained in the presence of a 1:20 dilution of hybridoma antibody 101-1 was resistant to neutralization by the selecting antibody. In contrast, the progeny of 16 plaques obtained in the presence of a 1:100 dilution of 101-1 antibody remained susceptible to neutralization by the selecting hybridoma antibody. Additional groups of variant viruses were selected by exposing CVS to seven different monoclonal antibodies. As shown in Table II, variants not neutralized by the selecting antibody were consistently obtained in experiments in which the neutralization index (the ratio of the titer of CVS in the presence of hybridoma antibodies to the CVS titer in the absence of antibody) was 10^4 or higher. Using a variety of hybridoma antibodies, the observed frequency of variants in individual glycoprotein epitopes was between $10^{-4.3}$ and $10^{-4.9}$ per infectious unit of CVS.

Antigenic Analysis of Variant Viruses. Five variant viruses, selected by titration of the cloned parental CVS-11 strain in the presence of an overneutralizing amount of

TABLE I

Selection of Variants of CVS Virus in the Presence of Hybridoma Antibody 101-1

Virus treatment	PFU/plate at virus dilution (log 10)						Infectivity titer (log 10)
	−1	−2	−3	−4	−5	−6	
							PFU/ml
None	C	C	C	>100	45	5	7.7
HAb 1:20	42 (16/16)*	5 (5/5)	0	0	0	0	3.7
HAb 1:100	C	>100	16 (0/16)	0	0	0	5.2

Dilutions of CVS were mixed with either 1:20 or 1:100 dilution of 101-1 hybridoma antibody (HAb) and added to CER monolayers; individual plaques were collected 4 d later. C, confluent.

* 42, number of plaques observed; (16/16), number of virus clones not neutralized by hybridoma antibody to number of clones tested.

hybridoma antibodies 101-1, 220-8, 231-22, 240-3, and 226-11, were tested in a neutralization test against nine hybridoma antibodies.

The following points are evident from Table III. First, the five CVS variants can readily be differentiated from each other by means of the hybridoma antibody panel. For instance, HAb 101-1 neutralizes all variant viruses except RV-101-1 and thus demonstrates the uniqueness of RV-101-1. Similarly, RV 220-8 and RV 231-22 can be differentiated from all the variants and from each other by the HAb's 231-22 and 162-5, respectively. The same holds here for variants RV 240-3 and RV 226-11 as evidenced, for instance, by HAb 226-11 and 613-2. Second, the variants seem to form two distinct groups: the variant group comprising RV 101-1, RV 220-8, and RV 231-22 exhibit changes that prevented several of the top four antibodies (101-1, 220-8, 162-5, 231-22) from neutralizing the variants but did not reduce detectably the neutralizing potency of five other antibodies (240-3 through 194-2). In contrast, the changes exhibited by the variant group RV 240-3 and RV 226-11 did not affect the neutralizing potency of the top four antibodies but abolished or reduced the neutralizing potency of the bottom five antibodies of the hybridoma panel. The latter finding suggests that the changes in the first and second variant group occurred in distinct antigenic sites of the viral glycoprotein which seem to vary independently from each other. These sites will be referred to in the following as I (modified on variant RV 101-1, RV 220-8, and RV 231-22) and II (RV 240-3 and RV 226-11).

Additional support for the notion of the independence of glycoprotein sites I and II comes from the demonstration (Table IV) that no variants were observed when CVS virus was titrated in the simultaneous presence of two hybridoma antibodies, one (101-1) directed against site I and the other (240-3) against site II. Given the frequency of variants ($10^{-4.1}$ and $10^{-4.2}$) in the epitopes delineated by the above

TABLE II

Frequency of CVS Variants

HAb used for selection of variants	Neutralization index (log 10)	Variants per plaques analyzed*	Frequency of variants‡
101-1	4.3	21/21	$10^{-4.3}$
	3.5	0/14	ND§
220-8	4.5	3/3	$10^{-4.5}$
226-11	4.5	3/9	$10^{-5.0}$
231-22	4.2	4/5	$10^{-4.3}$
240-3	4.5	1/1	$10^{-4.5}$
162-5	4.3	2/2	$10^{-4.3}$
194-2	4.0	3/8	$10^{-4.4}$
613-2	3.3	0/2	ND

Dilutions of CVS were mixed with 1:5 dilution of the respective hybridoma antibody (HAb) except in case of 101-1 when 1:100 dilution was also used, and the mixture was added to CER monolayer. From plates where well separated plaques were observed, virus clones were grown out and tested for neutralization by HAb used for their selection. Neutralization index was determined by dividing number of PFU/ml of virus mixed with normal medium over number of PFU/ml of virus mixed with antibody.

* Number of virus clones not neutralized by the HAb used for their selection to total number of virus clones isolated.

‡ Number of variants per infectious unit of CVS.

§ ND, not determined.

TABLE III

Neutralization of CVS Standard and CVS Variants by Different Hybridoma Antibodies

Hybridoma clone	Neutralization index of hybridoma antibodies reacting with parental virus and virus variants (log 10)					
	Parent CVS-11	RV 101-1	RV 220-8	RV 231-22	RV 240-3	RV 226-11
101-1	4.2	0	4.0	4.0	>4.0	>4.0
220-8	4.3	0	0	0	>4.0	>4.0
162-5	4.3	0	0	3.3	>4.0	>4.0
231-22	4.3	4.5	0	0	>4.0	>4.0
240-3	4.0	4.5	4.0	4.0	0	0
226-11	>4.0	>4.0	>4.0	>4.0	0	0
613-2	3.3	>3.0	>3.0	>3.0	>3.0	0
194-2	4.2	4.5	>4.0	4.0	>4.0	3.0
248-2	4.0	>4.0	>4.0	>4.0	>4.0	4.0

Dilutions of CVS and variant strains mixed with 1:5 dilution of hybridoma medium were used to infect CER cells.

TABLE IV

Attempts at Selecting a CVS Variant after Exposure to a Mixture of Two Hybridoma Antibodies

Hybridoma antibody	Neutralization index (log 10)
None	0
101-1	4.1
240-3	4.2
101-1 and 240-3	>7.4

Dilutions of CVS were mixed with 1:5 dilution of hybridoma antibody 101-1 and 240-3, respectively, and with equal mixtures of the two hybridomas before infecting CER monolayers.

antibodies, the expected frequency of variants in both epitopes should equal the product of the individual variant frequencies ($10^{-8.3}$) if the two epitopes change independently from each other. Although the expected frequency of double variants was one log below the level of the detectability of the above experiment, the failure to observe a double variant among $10^{7.4}$ infectious units of CVS is compatible with the independent mutability of the glycoprotein sites I and II.

Analysis of Variants in Protection Experiments In Vivo. The results shown in Fig. 1 indicate that the mice immunized with CVS-11 vaccine were protected against challenge with CVS-11 virus and against challenge with the variants RV 231-22 (site I) and RV 240-3 (site II). In contrast, mice immunized with variant vaccines were protected only when challenged with the homologous variant; they remained susceptible, however, to challenge with either CVS-11 virus or variants of the heterologous site. Although the mortality ratio of mice vaccinated with variants and challenged with the parental virus or variants was, at some dilutions of challenge virus, lower than in unvaccinated control mice, inoculation with dilutions 10^{-1}–10^{-3} of the challenge virus often caused the death of all vaccinated mice (Figs. 1 and 2). Thus, vaccination of mice with RV 240-3 (site II) did not result in sufficient protection against challenge with CVS-11 or RV 231-22 (site I) (Fig. 1). Conversely, vaccination with RV 231-22 or RV 101-1 (both site I) produced insufficient protection against

challenge with CVS-11 or a variant in the site II but protected well against variants in the I site (Figs. 1 and 2).

To investigate whether the different degree of cross-protection observed in vivo was related to differences in the humoral immune response elicited by CVS-11 and variant vaccine strains, the sera obtained from vaccinated mice before challenge were tested for their neutralizing potency against the viruses used for subsequent challenge in vivo. The results shown in Table V clearly demonstrate that the prechallenge sera are not able to differentiate clearly between homologous and heterologous viruses.

Variants of Street Rabies Virus. We have described elsewhere (6) the great diversity in antigenic specificities of field strains of rabies virus which could be demonstrated by means of a panel of either antinucleocapsid or antiglycoprotein monoclonal antibodies. Because mutant virus vaccines failed to protect mice against challenge with parental virus and other mutants, it became imperative to investigate whether vaccination with standard fixed virus vaccine protected against challenge with street viruses isolated directly from humans who had died of rabies, some of whom had received the complete course of postexposure vaccinations.

Before undertaking the protection test, it was necessary to obtain an adequate number of samples of street virus of human origin and to perform, by means of monoclonal antibodies, a comparative antigenic analysis between field strains and fixed viruses used for vaccine production. Eight coded samples of human-derived virus isolates were processed as described in Materials and Methods. Brain impressions from mice that had died after infection with the original material were examined by FA initially with a panel of three hybridoma antibodies (Table VI) to rule out the possibility that not all of the isolates were rabies virus. In the test we have included in addition to the eight samples of street viruses, impression smears of mouse brains infected with two strains of fixed virus, PV-11 and CVS-DEV (Table VI) used for the production of vaccines employed for immunization of man in this and other countries.

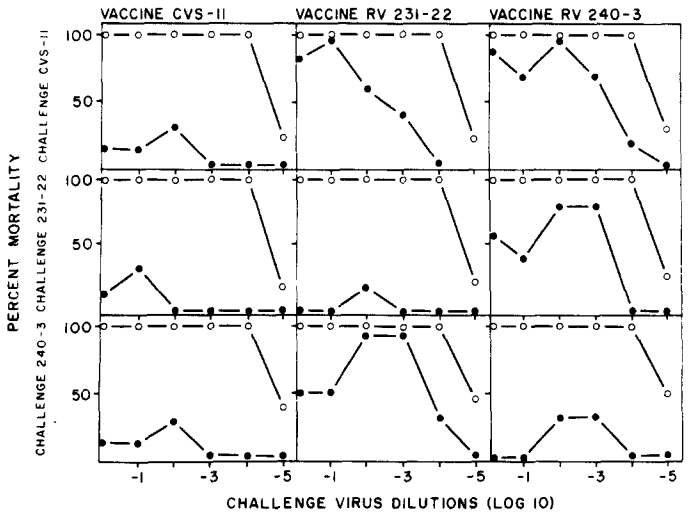

FIG. 1. Protection by parental and variant vaccines against challenge with parental and variant viruses. Percent mortality after challenge with indicated virus dilutions. ●, vaccinated mice; ○, unvaccinated controls.

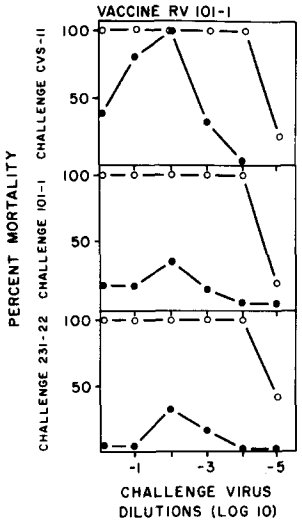

FIG. 2. Protection by one variant vaccine against challenge with parental virus, by homologous virus, and by another variant of the same epitope. Percent mortality after challenge with indicated virus dilution. ●, vaccinated mice; ○, unvaccinated controls.

TABLE V

Cross-Neutralization between Parent CVS-11 and Variant Strains Derived from CVS-11 by Polyclonal Antibodies

Serum from mice immunized with	Virus neutralizing antibody titers against virus strains			
	CVS-11	RV 231-22	RV 101-1	RV 240-3
CVS-11	2,400	800	800	800
231-22	800	800	800	800
101-1	2,400	2,400	6,000	2,400
240-3	2,400	2,400	2,400	2,400

Dilutions of sera from vaccinated mice obtained at the time of challenge (see text) were mixed with 100 ID_{50} of CVS-11 and variant strains and the mixture added to BHK-21 monolayer. Final dilution of antibody reducing the number of fluorescent foci by 50% was considered as end-point titration.

As shown in Table VI all viruses (representing eight human isolates and two fixed viruses) react with antibodies 502-2 and 103-7, and none reacts with antibody 422-5. Antibody 502-2 has been shown previously (6) to react with all strains of rabies and rabies-related viruses, antibody 103-7 with rabies viruses only, and antibody 422-5 with rabies-related viruses but not with rabies viruses. Thus, the results of the present assay indicate that all the human isolates were indeed rabies viruses. Results of the second part of the test permitted us to subdivide the street virus strains into three groups (1, 4, 5, 7, 8; 2, 6; 3) on the basis of antigenic properties of their nucleocapsids. In addition to the results shown in Table VI, 12 other antinucleocapsid hybridomas used in the FA test gave positive reactions with all the street and fixed viruses.

More striking differences between the eight street strains and the two fixed viruses were observed in the neutralization test with a panel of 14 antiglycoprotein hybridoma antibodies. As shown in Table VII, antigenic analysis of the street virus isolates and

TABLE VI

Analysis of Human Rabies Virus Isolates and Vaccine Strains with Anti-Nucleocapsid Monoclonal Antibodies

Hybridoma clone	Results of FA staining of human rabies virus isolates numbers			Results of FA staining of vaccine strains	
	1, 4, 5, 7, 8	2 and 6	3	PV-11	CVS-DEV
502-2*	+	+	+	+	+
103-7*	+	+	+	+	+
422-5*	0	0	0	0	0
377-3	+	+	+	+	0
120-2	+	+	0	+	+
237-3	0	+	+	+	+
102-27	0	+	+	+	+
364-11	0	+	+	+	+
390	0	+	+	+	+

An additional 12 antibodies reacted positively with all virus strains tested. Smears of brain cells of mice dying after intracerebral inoculation with respective virus strains were stained by anti-mouse fluorescein conjugate after incubation with hybridoma antibodies. +, positive fluorescence. 0, no fluorescence.

* Used for identification of isolates or rabies viruses.

of the two fixed viruses allows identification of six patterns of reactivity of the hybridoma antibodies with glycoprotein antigens expressed by the eight samples of street virus on the one hand and the fixed viruses on the other hand. These results further permitted grouping street virus isolates 4, 5, and 7 into one group (A), 1 and 8 into another group (B), 2 and 6 into the third group (C), and 3 alone representing the fourth group (D). It is evident also that each of these street virus groups differed antigenically from either one of the fixed virus strains.

To confirm on a more quantitative basis cross-reactivity between antigens recognized by hybridoma antibody 194-2 (Table VII) serial threefold dilutions of the antibody were mixed with 20–100 focus-forming units of strains 1, 3, 5, 6, and PV-11, respectively and the mixtures added to monolayers of BHK-21 cells. The antibody titers determined by rapid fluorescent focus-inhibition technique (13) were the same against each of the five virus strains.

Identification of the origin of street viruses (Table VIII) confirmed the validity of their classification on the basis of antigenic properties of their glycoproteins. Thus, strains 5 and 7 represent two samples of rabies virus isolated from the same patient, and strains 1 and 8 are samples of rabies virus isolated from brains of the donor and the recipient, respectively, of a corneal transplant (16).

Fixed Virus Vaccine Strain Does Not Give Uniform Protection against Street Virus Variants. Groups of mice were inoculated with a vaccine produced from strain PV-11 and then challenged intracerebrally with dilutions of homologous virus or of street viruses 2, 5, 6, and 7, respectively. Only the latter street virus samples were available in large enough concentrations at an early passage level in mice to be used in serial dilutions for challenge of vaccinated mice. Fig. 3 shows that vaccination with PV-11 protected against challenge with homologous virus as well as with street viruses 2 and 6. Conversely, challenge with street virus 7 and 5 resulted in 40 and 20–40% mortality,

TABLE VII

Analysis of Human Rabies Virus Isolates and Vaccine Strains with Selected Anti-Glycoprotein Monoclonal Antibodies

Hybridoma clone	Results of neutralization test with human rabies virus isolates numbers				Results of neutralization with vaccine strains	
	4, 5, 7 (A)	1 and 8 (B)	2 and 6 (C)	3 (D)	PV-11	CVS-DEV
194-2	+	+	+	+	+	+
248-2	+	+	+	+	+	+
613-2	+	+	+	+	+	+
101-1	+	+	+	+	0	0
162-5	+	+	+	+	0	0
110-3	0	+	+	+	+	+
507-1	0	+	+	+	+	+
509-6	0	+	+	+	+	+
528-2	0	+	+	+	+	+
231-22	0	0	+	+	+	+
120-6	0	0	+	+	+	+
176-2	0	0	+	+	0	+
193-2	0	0	+	+	0	+
503-1	+	0	0	+	0	0

An additional 10 antibodies did not react with strains of groups A–D. 100 tissue culture-infective doses of virus mixed with 1:5 dilution of respective hybridoma antibody were added to BHK-21 monolayers, and the number of foci of virus-infected cells was determined by FA staining. 0, >50% of cells showing fluorescence—no neutralization. +, no cells showing fluorescence—positive neutralization.

respectively, of vaccinated mice, regardless of the challenge dose used. Sera drawn from vaccinated mice at the time of challenge neutralized the PV-11 (vaccine) strain at a dilution of 1:7,200, and the strain 7, which caused 40% mortality in vaccinated mice, at a dilution of 1:2,400. Mortality resulted from rabies infection as evidenced by the presence of rabies antigen in the brain (FA staining) and by recovery of rabies virus after intracerebral inoculation of brain homogenate of diseased mice into naive mice.

Discussion

As has been observed previously with influenza (17, 18) and parainfluenza virus (J. W. Yewdell, Personal communication.), the present study shows that rabies virus variants can readily be selected in vitro from a cloned parental virus seed (CVS-11) if the latter is grown in the presence of a concentration of monoclonal antibody able to neutralize at least $10^{4.3}$–10^{5} infectious units of parental CVS virions. Rabies variants selected in this way can be characterized, briefly, as follows: first, variants in single glycoprotein epitopes (viz., antigenic structure delineated by the combining site of an individual monoclonal hybridoma antibody) are present in cloned rabies virus seed at a frequency of $10^{-4.3}$–10^{-5}. Although final proof (comparison of amino acid sequences of parental and variant glycoprotein) is still lacking, the high frequency of occurrence of the variants suggests that they represent single point glycoprotein mutants of the parental CVS virus. Second, the antigenic change exhibited by these variants prevents the monoclonal antibody used for variant selection from neutralizing the heterologous

TABLE VIII

Origin of Street Viruses Isolated from Human Brains

Street number		Group*
4	Oklahoma, 1979, exposure unknown	
5	Maryland, 1976, infection by bat bite‡	A
7	The same as 5	
1	Oregon, 1978, exposure unknown; donor of corneal transplant for case 8	
8	Idaho, 1978; recipient of corneal transplant from case 1	B
2	Texas, 1976, infection by dog bite in Mexico	
6	Texas, 1976, infection by dog bite in Mexico§	C
3	Minnesota, 1974, infection by cat bite‖	D

* See Table VII.

‡ Patient received human rabies immune globulin (HRIG) and 21 doses of vaccine starting from day 2 after exposure.

§ Patient received HRIG and vaccination after development of symptoms.

‖ Patient received 14 doses of vaccine starting 3 wk after exposure.

Note: all other cases were not treated.

variants but does not affect detectably the neutralizing potency of many other monoclonal antibodies or of antirabies antisera. Thus, the antigenic changes exhibited by the variants represent minor modifications of the overall antigenicity of the parental glycoprotein.

The above findings clearly demonstrate that rabies virus has a similar potential as influenza to undergo antigenic variation under suitable experimental conditions in vitro. However, unlike influenza, the existence of antigenic variants of rabies in nature has only become fully apparent when monoclonal antibodies were applied in the analysis of field strains isolated in different parts of the world (6). The present study also shows that the virus isolated from seven fatal cases of human rabies represent antigenic variants. It is noteworthy, that a complete postexposure vaccination treatment had been given to case 5 (7) (Table VIII) of these fatal rabies cases. Thus, the question could be raised whether the inability of standard fixed virus vaccine to protect rabies-infected individuals may be caused by antigenic variation of street virus.

The epidemiologic and pathogenic significance of antigenic variation in rabies was assessed first in cross-protection experiments using the variants isolated under controlled conditions in vitro. It was found that mice immunized with one variant and showing the presence of high titer of cross-reacting antibodies in their sera could not withstand challenge with another variant or with the parental virus. Only variants such as RV 101-1 and RV 231-22 (Table III) which could be paired in group I showed reciprocal cross-protection in mice.

It has been observed previously (19) that serum with neutralizing titer of 1:300-1:1,000 (2–6 IU) antibody protected all mice infected intracerebrally with the CVS strain. The discrepancy between those results and the present finding cannot be satisfactorily explained unless we postulate that only humoral antibodies of certain defined anti-glycoprotein specificities protect in vivo or alternately, that factors other than humoral immunity play a decisive role in resistence of animals against intracerebral challenge with rabies.

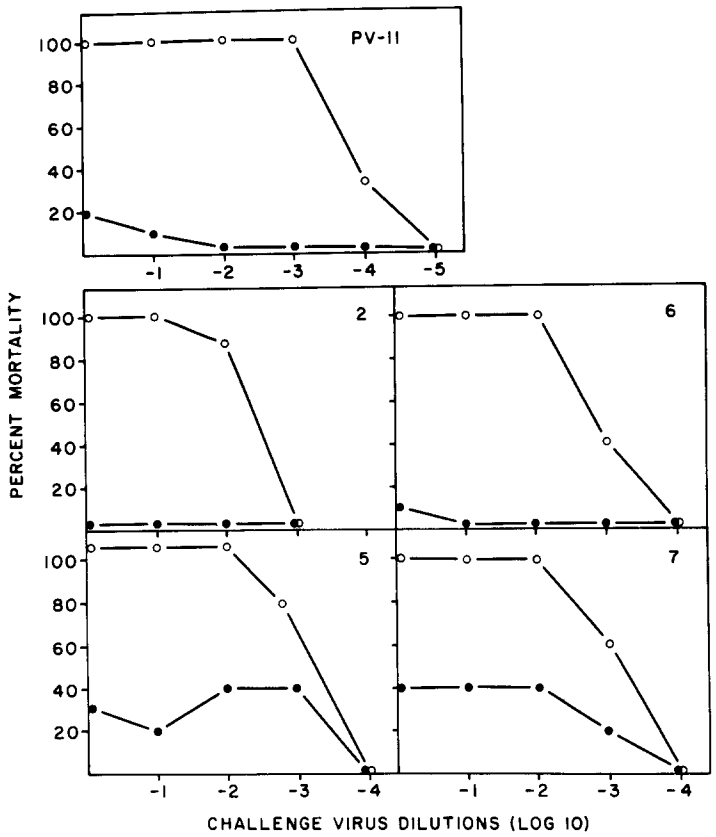

FIG. 3. Protection by vaccine prepared from PV-11 virus against challenge with homologous virus and against challenge with four street strains of rabies virus. Percent mortality after challenge with indicated virus dilutions. ●, vaccinated mice; ○, unvaccinated controls.

Failure of cross-protection between variants, coupled with the high frequency of variants among the street strains of rabies isolated from fatal human cases, pose the question of whether mice vaccinated with standard vaccine prepared from fixed viruses such as PV-11 or CVS-DEV would be protected against challenge with street virus representing different variant groups.

As was shown in Fig. 3, vaccination of mice with vaccine produced from PV-11 strain protected mice completely against lethal challenge with either homologous or the 2 or 6 street virus variants of group C, but only partially protected mice challenged with street virus 5 and 7 of group A. The latter results are rather hard to interpret because ~40% of vaccinated animals died after challenge with either street virus 5 or 7 regardless of whether they were challenged with undiluted virus or with 10^{-1}, 10^{-2}, or 10^{-3} (in the case of virus 7 challenge) dilutions of the virus suspension. This could hardly be explained by the existence of a mixture of two rabies populations in the challenge inoculum. It is possible that individual responses to vaccination of mice may have differed and that if inbred strains of mice instead of the ICR stock were used in the same type of experiment, the challenge results would become more understandable.

The fact that vaccine prepared from variants obtained by single point mutation cannot protect mice against challenge with either parental or another variant virus (Figs. 1 and 2), and that relatively large numbers of street virus variants exist in different parts of the world (6) and within a group of seven strains of viruses isolated from fatal human cases of rabies in the United States and Mexico (Table VIII) poses a question of whether the method of selection and of testing rabies strains for vaccine production for preventive and postexposure treatment of animals and man is correct.

Rabies vaccines are produced in many countries. No more than three strains of fixed virus, two of which are derived from the original Pasteur strain, are used for production of vaccines throughout the world (20) regardless of whether the vaccines are made in animal brain, duck embryo, or tissue culture.

Evaluation of the antigenic potency of the rabies vaccine is also a standardized procedure (21) adopted by most countries of the world. Mice are immunized with the vaccine and then challenged with a strain of fixed virus, usually the CVS, distributed from one stock by an international organization (20). CVS is a fixed strain of rabies also derived from Pasteur's strain; thus antigenicity of one derivative of the Pasteur strain is checked by challenge of mice with another derivative of Pasteur's strain, and this vaccine is used for protection of men and animals against street viruses which may, in different areas of the world, represent variants expressing antigens which do not cross-react with either of the strains of the fixed viruses involved in the vaccine evaluation procedure. Only once in the modern history of rabies research has a potency test been developed for a live virus animal vaccine (22) in which vaccinated animals were challenged with street virus instead of fixed virus. In spite of its successful applications to the evaluation of vaccines (23), this type of potency test had to be abandoned because of opposition by vaccine manufacturers and licensing authorities to its use.

In the light of results presented in this paper, however, the currently employed vaccine potency test should be immediately changed by modifying it through challenge of vaccinated animals with a street virus prevalent in a given geographical region. If vaccinated mice succumb to challenge with the virus, the existing vaccine should be replaced by one which uses fixed virus-expressing antigen(s) which evoke in the vaccinated individual an immune response capable of protecting it from exposure to street viruses in that particular region. Selection of fixed viruses tailored to the local needs may not be a simple matter, but then, the number of street virus variants in any given area may not be as great as feared. For instance, the two Mexican isolates reported in this paper, the two isolates from foxes in France and the Federal Republic of Germany (6), and two human isolates from U. S. S. R. (6) could be paired, on the basis of their reactivity with monoclonal antibodies, as apparently identical variants of rabies representing a certain geographical region. Moreover, further studies along the lines presented in this paper may lead to the development of methods permitting relatively rapid identification of antigenic sites of fixed virus strains which would engender the broadest possible protective response to challenge with a large number of variants of street virus.

Summary

Antigenic variants of CVS-11 strain of rabies virus were selected after treatment of virus populations with monoclonal antibodies directed against the glycoprotein

antigen of the virus. These variants resisted neutralization by the hybridoma antibody used for their selection. Two independently mutating antigenic sites could be distinguished when five variants were tested with nine hybridoma antibodies. The frequency of single epitope variants in a cloned rabies virus seed was ~1:10,000. Animals were not or only partially protected when challenged with the parent virus or with another variant, but were fully protected against challenge with the virus used for immunization. Variants were also detected among seven street viruses obtained from fatal cases of human rabies. Animals immunized with standard rabies vaccine were protected against challenge with some but not all street rabies variants. A comparative antigenic analysis between vaccine strain and challenge virus by means of monoclonal antiglycoprotein antibodies showed a slightly closer degree of antigenic relatedness between vaccine and challenge strain in the combinations where vaccination resulted in protection. It remains unknown, however, whether these apparently minor antigenic differences in the glycoproteins account for the varying degrees of protection. The results of this study clearly indicate that the selection of vaccine strains and the methods used to evaluate the potency of rabies vaccines need to be revised.

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