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Immunology

Monoclonal antibodies against rabies virus produced by somatic cell hybridization: Detection of antigenic variants

(mouse hybridomas)

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ABSTRACT **Somatic cell hybrids (hybridomas) between mouse myeloma cells and spleen cells derived from BALB/c mice immunized with inactivated rabies vaccine were found to produce antibodies to rabies virus. Monoclonal antibodies with different specificities were obtained either from the mass culture directly after fusion or from clones derived from a single-cell cloning procedure. Several strains of fixed or street rabies virus were analyzed by virus neutralization procedures which demonstrated differences in their antigenic composition. Hybridoma antibodies were able to protect experimental animals from lethal rabies virus infection.**

Antibodies produced in animals immunized with the nucleocapsid fraction of rabies virus crossreact with nucleocapsids of all strains of rabies but not with coat proteins (1, 2). Antibodies produced by immunization of animals with whole virions or with the glycoprotein fraction can barely detect antigenic differences in coat proteins of different strains of rabies virus (2, 3).

Because monoclonal antibodies produced by somatic cell hybrids (hybridomas) against influenza A virus (4, 5) detect even minor antigenic differences among variants of the same strain of virus (5), it should be possible to produce hybridomas expressing different antibody specificities for various rabies strains.

In the present study we have investigated the feasibility of production of antirabies antibodies by hybridomas formed by fusion of P3 × 63Ag8 myeloma (6) with splenocytes from rabies virus-immunized mice. Antirabies antibodies produced by hybridomas were analyzed for their specificities in various assays and used to protect mice infected with rabies virus.

MATERIALS AND METHODS

Virus Strains. Clone-purified ERA, PM, CVS, and Flury HEP strains of rabies virus were propagated in BHK-21 cell culture monolayers as described (7). Nine additional strains of rabies virus were obtained from different sources, as indicated in Table 3, and adapted by us to growth in BHK-21 cell cultures by described techniques (7).

Vaccine. Concentrated, purified, β-propiolactone-inactivated rabies vaccine was prepared from the ERA strain of virus according to reported techniques (8). The antigenicity of this vaccine preparation was 20 times the value of the standard WHO reference vaccine (9).

Mice. Ten- to 12-week-old female BALB/c mice (Jackson Laboratories, Bar Harbor, ME) received a primary immunization of 0.1 ml of vaccine by intraperitoneal inoculation and a booster inoculation of the same vaccine diluted 1:5 3–4 months later by intravenous inoculation.

Production of Hybrid Cells. Splenocytes from rabies-immunized mice were fused with P3 × 63Ag8 mouse myeloma cells (6) as described (4, 5). After fusion, cells were seeded in individual wells of Linbro FB 16-24 TC plates in hypoxanthine/aminopterin/thymidine medium (10) at a concentration of approximately 10^6 spleen cells per well and treated as described. Cloning of hybrid cultures has been described (4).

ANTIBODY ASSAY

Radioimmunoassay (RIA). This test was performed as described (11) except that BHK-21 cells infected with the ERA strain of rabies virus and fixed in 10% formaldehyde solution were used instead of VSW cells. Total antibodies bound to the viral immunoadsorbent were detected by means of ^{125}I-labeled rabbit anti-mouse F(ab')$_2$ antibodies, provided by W. Gerhard of the Wistar Institute. The reaction was considered to be positive when the number of ^{125}I counts obtained with the test sample was at least 3 times that obtained with the control sample which consisted of medium only.

Virus-Neutralizing Antibody Determination (VN). Virus-neutralizing antibodies were measured by the rapid fluorescent focus inhibition technique (12) against the ERA, CVS, and HEP strains of virus. Titers are expressed in international units (IU) of rabies antibody (13).

The neutralizing effect of hybridoma tissue culture medium on different strains of rabies virus was also evaluated by determination of virus neutralization index. Dilutions (1:10) of virus (0.1 ml) were mixed in eight-chamber Lab-Tek tissue culture chamber/slides (Lab-Tek Products, Westmont, IL) with equal volumes of 1:3 dilution of the tissue culture medium to be tested and incubated at 37° for 1 hr. Indicator BHK-21 cells (4×10^4 per well) were added in 0.2 ml of medium, and cultures were incubated at 37° for 48 hr. After fixation and fluorescent antibody staining, preparations were examined under a UV microscope, and the percentage of infected cells at each virus dilution was determined. The neutralization index was determined by comparing the number of infected cells in control cultures with the number of infected cells in cultures incubated with hybridoma medium. A difference of at least 1 order of magnitude (1.0 logarithm unit) was considered as evidence of virus neutralization.

Cytotoxic Antibody Test (CT). BHK-21 cells (5×10^6) infected in suspension with ERA, CVS, or HEP strain of virus at a multiplicity of infection (MOI) of 10 were incubated for 20 hr in T-25 Falcon tissue culture flasks in 5 ml of minimum essential medium. Infected cells were labeled with $Na_2{}^{51}CrO_4$

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Abbreviations: RIA, radioimmunoassay; VN, virus-neutralizing antibody determination; CT, cytotoxic antibody test; MF, membrane fluorescent antibody staining; NCF, nucleocapsid fluorescent antibody staining; MOI, multiplicity of infection; P_i/NaCl, phosphate-buffered saline.

as described (14) and adjusted to contain 1×10^4 cells in 0.05 ml. The uninfected control BHK-21 cells were treated similarly.

Serial dilutions of antibody (hybridoma medium) (0.05 ml per well) were prepared in Microtest II tissue culture plates (Falcon 3040); ^{51}Cr-labeled cells (0.05 ml) and fresh guinea pig complement diluted 1:60 (0.05 ml) were added to each well. Plates were incubated at 37° in a CO_2 incubator for 4 hr. Control preparations included a series in which antibody or complement was omitted and replaced by medium. All determinations were performed in triplicate. Half of the medium from each well was transferred into a small glass tube, and the radioactivity of each sample was assayed in a γ counter. Results were calculated after subtraction of background activity, and the reciprocal of the antibody dilution causing 20% increase over the background was considered to be the cytotoxic titer of a given antibody preparation.

Membrane (MF) and Nucleocapsid (NCF) Fluorescent Antibody Staining. BHK-21 cells infected with either ERA, CVS, or HEP virus at a MOI of 0.1 were seeded in eight-chamber Lab-Tek slides (4×10^4 cells per well) and incubated for 48 hr at 37°.

For MF staining, *unfixed* cultures were washed with phosphate-buffered saline (P_i/NaCl), treated with undiluted hybridoma tissue culture medium for 30 min, washed in P_i/NaCl, and stained for another 30 min with fluorescein-conjugated anti-mouse gamma globulin (Cappel Laboratories, Downingtown, PA) (Fig. 1, *left*).

For NCF staining, cells were fixed for 5 min in cold (4°) acetone and air dried. After UV microscope examination for detection of membrane antigen, slides were treated for the second time with hybridoma tissue culture medium and anti-mouse fluorescent conjugate and examined for detection of intracytoplasmic fluorescence (Fig. 1, right).

RESULTS

Production of Antirabies Antibody-Secreting Hybridomas. Hybrid cultures secreting antibody binding to rabies-infected cells in RIA were easily produced by fusion of mouse splenocytes with mouse myeloma cells as long as spleen cells were obtained from mice within a short interval (3–4 days) (83 of 96 cultures at 3 days and 20 of 24 cultures at 4 days) after booster inoculation with rabies vaccine. When spleens were removed from mice 10 days after booster inoculation, none of the 20 hybridomas was found to produce detectable levels of rabies antibody.

Specificity of Antirabies Antibodies Produced by Hybridomas. Of 83 hybridomas produced after fusion of splenocytes from rabies-immunized mice with P3 × 63Ag8 cells, 52 secreted antibodies binding in the RIA to BHK-21 cells infected with ERA virus. When these antibodies were assayed in VN, CT, MF, and NCF tests against ERA, CVS, and HEP strains of rabies virus, they displayed different specificities (Table 1). Of the 10 groups of hybridomas, 9 produced antibodies reacting in VN, CT, and MF assays; 1 group, comprising 22 hybridomas and represented by hybridoma 104, was reactive only in the

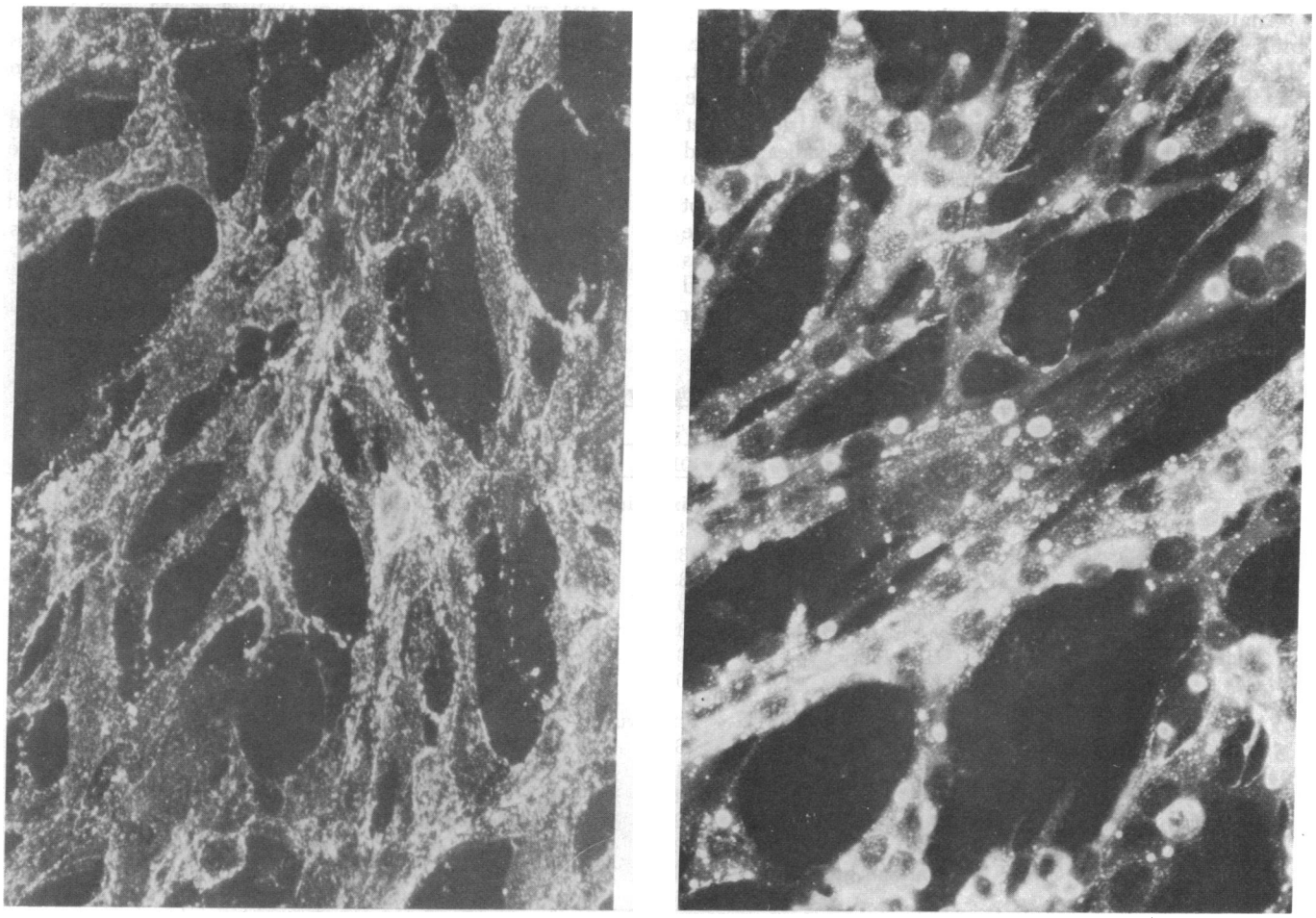

FIG. 1. Indirect fluorescent antibody staining of rabies (ERA)-infected BHK-21 cells. (*Left*) Unfixed cells; membrane fluorescence. (×250.) (*Right*) Fixed cells; nucleocapsid fluorescence. (×250.)

Table 1. Antibodies produced by antirabies hybridomas

Hybridoma cultures: No. secreting antibodies*	Hybridoma cultures: Representative	Reactivity against various virus strains in different assays: ERA strain VN	ERA strain CT	ERA strain MF	ERA strain NCF	CVS strain VN	CVS strain CT	CVS strain MF	CVS strain NCF	HEP strain VN	HEP strain CT	HEP strain MF	HEP strain NCF
7	101	>6.0	240	+	−	>6.0	>240	+	−	3.0	240	+	−
2	181	3.0	180	+	+	2.5	30	+	+	1.0	180	+	+
2	194	>6.0	>240	+	−	>6.0	240	+	−	<0.1	<10	−	−
11	120	0.4	240	+	−	<0.1	<10	−	−	0.4	30	+	−
2	103	0.4	240	+	+	<0.1	<10	−	+	>6.0	30	+	+
1	132	1.0	180	+	−	<0.1	<10	−	−	<0.1	<10	−	−
2	193	0.4	10	+	+	<0.1	<10	−	+	<0.1	<10	−	+
1	152	<0.1	<10	−	−	<0.1	<10	−	−	2.5	80	+	−
2	159	<0.1	<10	−	+	<0.1	<10	−	+	3.0	NT	+	+
22	104	<0.1	<10	−	+	<0.1	<10	−	+	<0.1	<10	−	+

* Antirabies antibodies determined by RIA.

NCF assay. Hybridomas represented by cultures 101 and 181 crossreacted in VN, CT, and MF assays with all three strains of rabies virus. Antibodies produced by hybridomas represented by culture 194 reacted with ERA and CVS strains, whereas those produced by groups 120 and 103 reacted with ERA and HEP strains. Hybridoma groups 132 and 193 reacted only with the ERA strain (193 did so weakly), and groups 152 and 159 reacted only with HEP virus. VN-, CT-, and MF-reactive antibodies secreted by hybridoma groups 181, 103, 193, and 159 were also reactive in NCF tests with all three rabies strains.

Analysis of Antigenic Relationship among Strains of Rabies Virus by Means of Hybridoma Antibodies. The results shown in Table 2 indicate that the Kelev virus, a nonvirulent, fixed strain, was neutralized only by antibodies secreted by one hybridoma, and the recently discovered South African street virus (Duvenhage) was neutralized only by antibodies secreted by two hybridomas. In contrast, all street virus strains seem to be crossreactive in VN except for the AF strain which did not seem to react with antibody 193. Hybridoma antibodies were found to be quite heterogenous in VN assays with fixed strains of virus. For instance, the PM and CVS strains, both derived from the Pasteur strain, reacted differently from the Pasteur strain and from each other. Conversely, the SAD virus and its derivative ERA reacted in an identical fashion with antibodies secreted by the same hybridomas. Finally, the nonvirulent HEP strain was found to be crossreactive with the Kelev strain in the VN with antibody 120. In addition, however, HEP was neutralized by antibodies secreted by three other hybridomas.

Cells infected with any virus, regardless of origin, showed intracytoplasmic fluorescence after fixation and exposure to antibodies produced by hybridoma 104 (NCF assay), even though none of the viruses reacted with the same hybridoma in VN. This confirms previous results that, although the hybridoma antibodies easily distinguish strains of fixed rabies virus in VN, CT, and MF tests, they cannot distinguish between strains of rabies in the NCF assay.

Reactivity of Antirabies Antibodies Produced by Cloned Hybridomas. Three hybridomas—101, 120 and 103—were cloned, and the specificity of antibodies produced by individual clones was compared to the specificity of the antibodies secreted by the parental culture. All clones derived from hybridomas 101 and 120 showed the same specificities in VN, CT, and MF as the parental cultures (data not shown). The cloning of hybridoma 103 is of special interest because the antibodies pro-

Table 2. Crossreactivity between strains of rabies virus of various origins determined in neutralization test with hybridoma antibody

Origin of strain: Host	Country	Prototype	Derivative	Neutralization index (by logarithm units): 101	110	194	120	103	193	104
				Fixed virus						
		SAD		>3.0	>3.0	>3.0	2.5	3.0	1.5	0
Dog	USA		ERA	>3.0	3.0	>3.0	2.5	3.0	1.0	0
		Pasteur		>3.0	>3.0	>3.0	1.0	3.0	1.0	0
Cow	France		PM	0	>3.0	>3.0	3.0	3.0	0	0
			CVS	>3.0	2.0	>3.0	0	0	0	0
Man	USA	HEP Flury		>3.0	>3.0	0	2.0	3.0	0	0
Dog	Israel	Kelev		0	0	0	>3.0	0	0	0
				Street virus						
Dog	USA	NYC		>3.0	3.0	3.0	2.0	>3.0	1.0	0
Bat	USA	UD		1.5	>3.0	>3.0	2.0	>3.0	1.5	0
	Brazil	DR		>2.0	1.5	>2.0	1.5	1.0	1.0	0
Fox	France	AF012.0		2.0	>2.0	2.0	2.0	0	0	
Dog	Rwanda	RD		>3.0	>3.0	>3.0	2.5	>3.0	2.5	0
Man	South Africa	Duvenhage		0	0	1.5	1.5	0	0	0

Intracytoplasmic fluorescence staining in the presence of medium from hybridoma 104 (Table 1) indicates the presence of common nucleocapsid antigens in cells infected with all strains listed in this table.

Table 3. Results of cloning of hybridoma 103 (see Table 1)

	No. antirabies antibody	Fluorescent methods			
		MF only	NCF only	MF and NCF	RIA* only
1st cloning	0/20	11/20	1/20	8†/20	0/20
Recloning					
C1-1	1/21	0/20	0/20	20/21	0/20
C1-2	1/19	0/19	0/20	15/19	3/19

Data shown as no. clones secreting antibody/total no. tested.
* No fluorescence; antibodies detected only in RIA.
† Two clones used for recloning.

duced by the mass culture reacted in VN, CT, and MF assays as well as in the NCF assay. As shown in Table 3, one clone in each experiment did not produce antibodies whereas the other clones in C1-1 and 15 of 19 in C1-2 produced antibodies reacting again in both MF and NCF. In addition, three clones produced in the C1-2 experiment reacted in neither MF nor NCF assay but were found to bind to rabies-infected cells in RIA.

Protection of Mice by Hybridoma Antibody. Cells of hybridoma mass cultures C-1 and B-1 and of clone 110-5 were implanted subcutaneously into BALB/c mice. Serum obtained from these mice within 2 weeks after implantation of the C-1 and 110-5 hybridomas showed a 100-fold higher concentration of rabies antibodies than did medium from tissue cultures of the same hybridoma. Five days after implantation of hybridomas, mice were challenged intracerebrally with a lethal dose of PM virus. Mice that were implanted with hybridoma cultures that secreted VN antibody survived viral challenge, whereas mice that carried hybridomas that did not secrete VN antibody were not protected (Table 4).

Table 4. Protection of mice against challenge by rabies virus by hybridoma antibodies

Hybridoma	No. of cells inoculated	VN	No. of mice protected/no. challenged
C-1	5×10^6	+	6/6
110-5	5×10^5	+	9/10
	5×10^4	+	6/6
B-1	5×10^6	−	0/6
−	None	−	0/6

DISCUSSION

Fusion of P3 × 63Ag8 myeloma cells with splenocytes obtained from mice immunized with ERA strain rabies vaccine resulted in production of large numers of antirabies antibody-producing hybrid cells. As in the case of hybridoma antibodies against influenza virus (4), antirabies antibodies secreted by clones derived from individual hybrid cultures in general showed identical reactivity. Thus, even though the original hybridoma may have been polyclonal, it seems that, in the course of a few cell generations, the progeny of a single clone overgrew the culture.

Antirabies antibodies produced by hybridomas expressed different specificities in interaction with various strains of rabies virus. For instance, antibodies produced by hybridoma 193 (Table 2) reacted only with three of seven strains of virus investigated, whereas those produced by hybridoma 120 reacted with six of seven strains. It was also possible to show that strains of fixed rabies virus fall into several antigenic groups in relation to their crossreactivity with antibodies produced by a given hybridoma. The reactivity of hybridoma antibodies in VN tests against strains of fixed virus ranged from a capacity to neutralize more than 1000 plaque-forming units of virus to none. Antibodies produced by immunization of animals could never detect such antigenic differences. Antigenic variations that occurred in the course of numerous passages of rabies virus of the same origin in different laboratories were detected in tests with the two Pasteur virus-derived strains, CVS and PM (Table 2). Conversely, the SAD-derived ERA strain showed identical crossreactivity in tests with antibodies produced by six hybridomas.

Although the strain least virulent for animals, Kelev, interacted with antibodies produced only by one of six hybridomas, the almost equally attenuated HEP Flury strain crossreacted with antibodies secreted by four hybridomas. The present repertoire of antibody specificities of hybridomas did not permit antigenic analysis of street virus strains because five of six strains studied crossreacted with antibodies produced by all hybridomas. The one exception was the Duvenhage strain recently isolated in South Africa, which differs biologically from other strains of rabies virus to such an extent that not classifying it as rabies virus has been proposed (15). This seems to be an unwarranted step because Duvenhage virus was neutralized by antibodies produced by two hybridomas, one of which, 120 (Table 2), recognized antigenic determinants of all but one strain of rabies.

Confirming, in general, results obtained with antibodies produced *in vivo* (1), hybridoma antibodies interacting with antigenic determinants on nucleocapsids of rabies virus crossreacted with all fixed and street virus strains. However, in contrast to the restricted specificities (see introduction) expressed by antinucleocapsid antibodies produced *in vivo*, we have isolated clones of hybridomas that expressed specificities showing crossreactivity between antigenic determinants on nucleocapsids and coat proteins. Thus, monoclonal antirabies antibodies produced by hybridomas recognized antigenic determinants: (*i*) of viral coat protein only, (*ii*) of viral nucleocapsid only, (*iii*) common to coat protein and nucleocapsid, and (*iv*) of unknown specificity recognized only by the binding of antibody to rabies-infected cells in RIA.

Finally, we were able to demonstrate that virus-neutralizing antibodies produced by hybridomas fully protected mice against the lethal effect of intracerebral injection of rabies virus.

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