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Antibody Response *In Vitro* to an Animal Virus: Production of Rabies Virus Neutralizing Antibodies by Mouse Cells in Culture

(spleen cells/rhabdovirus/macrophages/erythrocytes)

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ABSTRACT **Rabies virus neutralizing antibodies were produced *in vitro* by the exposure of mouse spleen cells to live and inactivated rabies virus suspensions and to sheep erythrocytes coated with rabies virus. These antibodies did not neutralize two other rhabdoviruses: Kern Canyon and vesicular stomatitis viruses, and were precipitable by treatment with an antiserum to mouse IgG. Removal of "glass-adhering" cells from mouse spleen cell suspensions abolished the antibody response, which could be restored by the addition of mouse peritoneal exudate cells, rich in macrophages.**

The production of neutralizing antibody against T2 bacteriophage in cultures of lymph node cells from normal rats was described some time ago (1). There have been no reports, however, on the initiation of primary antibody response *in vitro* by an animal virus, even though the interactions of animal viruses and cells of the immune system have been extensively investigated (2).

Here, we report the production of neutralizing antibodies against rabies virus by mouse spleen cells from normal mice exposed in tissue culture to rabies virus suspension and to rabies virus adsorbed on sheep erythrocytes.

MATERIALS AND METHODS

Antigens

Viral Antigens. Clonal isolates of ERA and HEP strains of rabies virus were propagated in monolayers of BHK/S13 cells grown in roller bottles according to a technique described elsewhere (3). Tissue-culture media infected with the ERA strain used in the test had an infectivity titer of 6×10^8 plaque-forming units (PFU)/ml.

Tissue-culture medium infected with HEP strain (infectivity of 4×10^8 PFU/ml) was concentrated by ultrafiltration; the concentrate was purified by centrifugation for 90 min at 35,000 $\times$ *g* (two cycles), and the virus was inactivated by ultraviolet (UV) irradiation (4). The concentration factor of this purified and irradiated preparation was 10 times the original volume of infected tissue-culture fluid.

Erythrocyte Antigens. A stock solution of sheep erythrocytes was obtained from Baltimore Biological Labs. Co., Cockeysville, Md. Mouse erythrocytes were obtained by heart-puncture of C57/BL6 mice. Erythrocytes were washed three times in phosphate-buffered saline (pH 7.4), and the final concentration of the suspension was adjusted to 0.5% of erythrocytes in RPMI (see *Media*).

For adsorption of virus, 1 ml of a 10% suspension of either sheep erythrocytes or mouse erythrocytes was mixed into 3 ml of tissue-culture medium containing virus of strain ERA (6×10^8 PFU/ml), and the mixture was kept at 0° for 1 hr. The cells were then sedimented by centrifugation, washed once with cold RPMI, and resuspended at a final concentration of 0.5% erythrocytes in RPMI. Infectivity of this preparation was 4×10^6 PFU/ml.

Precipitating Serum. Antiserum to mouse IgG (anti-mouse IgG) (kindly provided by Dr. L. Manson, The Wistar Institute), was prepared as follows: A goat was given an initial injection of 10 mg of purified mouse IgG (Pentex, Inc., Kankakee, Ill.) in complete Freund's adjuvant administered in several subcutaneous sites, and subsequently inoculated at weekly intervals with 10 mg of mouse IgG without adjuvant. Serum was obtained 7 days after the fifth inoculation. It was determined by radioimmunoassay that specific anti-mouse antibodies represented about 2.5% of the total IgG of this serum (L. Goldstein, The Wistar Institute, personal communication).

Cell cultures

Culture Media. Unless otherwise specified, RPMI 1640 medium (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 1% of L-glutamine (200 mM) and 10% fetal-calf serum was used throughout the experiments. Penicillin (100 units/ml) and streptomycin (100 μg/ml) were added to the complete medium (RPMI).

Spleen Cells. Spleens from 12- to 15-week old C57/BL6 inbred mice were obtained aseptically, minced with scissors, and gently passed through a stainless-steel screen (150 mesh); a rubber stopper was used as a pestle. The cell pulp was suspended in cold RPMI medium (3 ml per spleen) and layered over 2 ml of fetal-calf serum in a 40-ml conical centrifuge tube to allow large cell clumps to settle. The upper layer was transferred to another tube and centrifuged at 800 $\times$ *g* for 10 min. The supernatant fluid was discarded, and the cells were resuspended in a 0.83% solution of NH_4Cl in distilled water (3 ml per spleen). The mixture was kept in ice for 5 min. Then 2 ml of fetal-calf serum was introduced at the bottom of the tube with a Pasteur pipette and the cells were centrifuged at 400 $\times$ *g* for 7 min. The result was a clear pellet of spleen cells, free of red cells, referred to as unfractionated spleen cells. Their viability, measured by exclusion of erythrosin B, was usually about 80%.

Column Purification of Spleen Cells. An aliquot of unfractionated cells (containing about 2×10^7 cells per ml) sus-

TABLE 1. *Production of rabies virus neutralizing antibody by unfractionated mouse-spleen cells in tissue culture*

Exp. no.	Antigen added	Antibody formation: PFC* per 1 × 10^6 cells	Viral neutralization titer: Rabies	Kern Canyon	Vesicular stomatitis
1	Sheep erythrocytes	435	<2	<2	<2
	Sheep erythrocytes + live ERA strain	420	56	<2	<2
	Live ERA strain	48	16	<2	<2
	None	35	<2	<2	<2
2	Sheep erythrocytes	370	<2	—	—
	Sheep erythrocytes + live ERA strain	390	100	<2	<2
	Live ERA strain	—	36	—	—
	None	7	<2	—	—
3	Sheep erythrocytes	325	<2	—	—
	Sheep erythrocytes + live ERA strain	300	60	—	—
	Live ERA strain	—	20	—	—
	Inactivated HEP strain	—	10	—	—
	None	11	<2	—	—

* PFC, plaque-forming cells causing hemolysis of sheep erythrocytes. Mean value from 2–3 determinations; (—), not tested.

pended in a mixture of 50% RPMI and 50% serum from C57/BL6 mice was passed through a 140 × 20-mm column of siliconized glass beads (450-μm diameter), which had been equilibrated with the mouse serum–RPMI medium, at 37° over a period of 13 min (5). About 55% of the cells were recovered after filtration, and viability was increased to 85–90%. This filtrate of cells represented the "nonadherent" fraction of the spleen cell population (5), and is referred to as column-purified lymphocytes.

Macrophages. C57/BL6 mice were injected intraperitoneally with 2% starch suspension (1.5 ml) in $NaPO_4$–NaCl. 3 Days later, the animals were killed by cervical dislocation, and their peritoneal cavities were washed twice with RPMI medium. The fluids thus obtained were seeded in 1-liter Blake bottles (1.5 to 2 × 10^7 cells per bottle in 80 ml of medium). After 45 min of incubation at 37°, the unattached cells were removed and the cell monolayers were washed twice with RPMI medium. New medium was added, and the cells were incubated at 37° for at least 2 days before use. The cells were dispersed by treatment with a solution of 0.25% trypsin in 0.1% EDTA, and washed twice with RPMI medium before use.

Preparation of Cultures. Marbrook's vessels (6) containing 12 ml of RPMI medium in the outer tube were used for all experiments. In the inner tube, either 10^7 unfractionated spleen cells or column-purified lymphocytes were planted in 0.5 ml of RPMI medium; different antigens were immediately added in 0.05-ml aliquots. In experiments where macrophages and purified lymphocytes were used, the tubes were first seeded with 2 × 10^5 macrophage cells harvested from Blake bottles in 0.5 ml of medium. After 12 hours of incubation at 37° to allow the macrophages to adhere to the surface of the membrane covering the bottom of the inner tube, the supernatant medium was gently removed by suction and the tubes were seeded with 10^7 lymphocyte cells and antigens as above. All cultures were incubated for 5 days at 37° in a humidified incubator in 95% air–5% CO_2.

Assay procedures

Collection of Medium from Cultures. The medium from the inner tube of the Marbrook vessel was separated from the cells and heated at 56° for 60 min before use as a source of antibody in the virus neutralization test.

Infectivity Assays and Neutralization Tests. Stock solutions of the CVS-11 strain of rabies virus (7), Kern Canyon virus (8), and the Indiana strain of vesicular stomatitis virus were produced by propagation of the viruses in BHK-21 cells (9). The BHK/S13 cells were used for infectivity titrations by the plaque assay technique (10) for all viral strains.

The virus neutralizing activity of culture media was measured by the plaque reduction technique (11) on BHK-S13 cells in a neutralization test against 500 PFU/ml of the virus strain. The neutralizing titer of the medium was expressed as the reciprocal of the end-point dilution that caused a 50% reduction in the number of plaques.

Detection of Hemolytic Plaque-Forming Cells. Cells from cultures were resuspended in 1 ml of RPMI medium, transferred into centrifuged tubes, and, after washing with medium, used for the detection of hemolytic plaque-forming cells. A modification (12) of the Jerne technique was used. The results were expressed as plaque-forming cells/10^6 cells plated at the beginning of the experiment.

The viability of these cells was 40–55%.

RESULTS

The exposure of spleen cells to sheep erythrocytes coated with rabies virus resulted in the production of rabies neutralizing antibodies in the tissue culture medium in all the experiments (Table 1). In the same experiments, neutralizing antibodies were formed *in vitro* after exposure of spleen cells to a suspension of live ERA strain virus or to inactivated HEP-strain vaccine preparation, but the neutralizing titer was lower than after exposure to sheep erythrocytes coated with rabies virus. Spleen cells maintained in culture without exposure to antigen did not form antibodies against rabies virus. Exposure of spleen cells to either sheep erythrocytes or sheep erythrocytes coated with rabies virus resulted in the formation of the same number of plaque-forming cells on sheep erythrocyte monolayers.

Tissue culture medium containing rabies neutralizing antibodies produced by spleen cells after exposure to either sheep erythrocytes coated with rabies virus or to live rabies virus suspensions did not neutralize either Kern Canyon or vesicular stomatitis virus (Table 1).

To determine whether the rabies inactivating substance was indeed an antibody produced by mouse spleen cells, 1 ml of tissue culture medium of mouse spleen cells exposed to sheep erythrocytes coated with rabies virus (Exp. No. 3, Table 1) was mixed with 0.1 ml of anti-mouse IgG, and was

incubated for 1 hr at 37° and 6 hr at 4°. The resulting precipitate was centrifuged at 1000 × *g* for 30 min, and the supernatant was assayed for rabies neutralizing antibodies. The control medium was processed in the same manner, except that anti-mouse IgG was not added to the medium. As shown in Fig. 1, the rabies neutralizing capacity of the tissue culture medium was almost completely precipitated by anti-mouse IgG.

In order to evaluate the role of various cell elements in the production of rabies neutralizing antibodies *in vitro*, a suspension of spleen cells was passed through a column of glass beads, and the resulting purified lymphocytes were used for the production of rabies neutralizing antibodies in tissue culture.

As shown in Table 2, medium harvested from these purified cells did not contain rabies neutralizing antibodies after exposure to either sheep erythrocytes coated with rabies virus or to a suspension of rabies virus. The same type of cells also failed to form plaque-forming cells capable of lysing sheep erythrocytes.

However, when column-purified lymphocytes were supplemented with mouse peritoneal macrophages, rabies neutralizing antibodies were produced after exposure to either sheep erythrocytes coated with rabies virus or to a suspension of live rabies virus. The capability of these "mixed" cell cultures to react immunologically was confirmed by the production of plaque-forming cells after exposure to sheep erythrocytes alone or sheep erythrocytes coated with rabies virus.

An attempt was made to determine if rabies virus adsorbed on heterologous sheep erythrocytes exerts a greater stimulus for the antibody production *in vitro* than rabies virus adsorbed on homologous cells. In contrast to the results obtained with sheep erythrocytes, adsorption of the rabies virus on mouse erythrocytes did not enhance the immunogenicity obtained with a suspension of rabies virus alone.

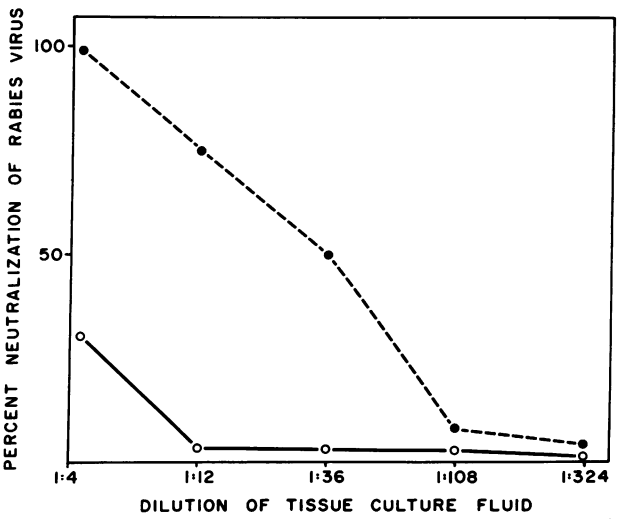

FIG. 1. Immunoprecipitation of rabies virus neutralizing antibodies by antiserum to mouse IgG. Tissue culture fluid containing antibody was mixed with the anti-mouse IgG. Immune complexes were sedimented, and the supernatant fluid (O——O) was evaluated for the presence of rabies antibodies by the plaque reduction technique. Tissue culture fluid from untreated control (●——●).

DISCUSSION

The preliminary data presented in this communication indicate that rabies virus is capable of initiating a primary antibody response in mouse spleen cells maintained *in vitro*.

TABLE 2. *Role of various cell elements in the production if rabies neutralizing antibody in tissue culture*

Exp. no.	Cells in culture (1×10^7)	Peritoneal macrophages added	Antigen added	Antibody formation: PFC* per 1×10^6 cells	Antibody formation: Rabies virus neutralization titer
1	Unfractionated spleen cells	None	Sheep erythrocytes	345	<4
			Sheep erythrocytes + rabies virus†	270	120
			Rabies virus	22	36
			None	18	4
	Column-purified lymphocytes	None	Sheep eryrhrocytes	3	<4
			Sheep erythrocytes + rabies virus	15	<4
			Rabies virus	16	<4
			None	4	<4
		2×10^5	Sheep erythrocytes	250	<4
			Sheep erythrocytes + rabies virus	165	100
			Rabies virus	3	40
			None	11	<4
2	Unfractionated spleen cells	None	Mouse erythrocytes + rabies virus	13	40
			Rabies virus	43	40

* PFC, plaque-forming cells.
† Strain ERA, live, was used.

The specificity of the reaction was confirmed in experiments that demonstrated that antibodies produced after exposure of mouse spleen cells to sheep erythrocytes neutralized rabies virus, but did not neutralize the immunologically unrelated Kern Canyon or vesicular stomatitis virus. The same specificity was shown by antibody produced by the reaction of mouse spleen cells with tissue culture medium infected with rabies virus grown in BHK-21 cells.

There have been no reports on the initiation of antibody synthesis *in vitro* by animal viruses, and it is not known whether there are other viral agents pathogenic for animals that have failed to evoke immune responses *in vitro*.

Viruses that replicate in cells of the immune system and functionally impair—or even destroy—them might have difficulty initiating antibody production *in vitro* by the very cells that are being infected. Among rhabdoviruses, vesicular stomatitis virus replicates in macrophages, and to a greater extent in stimulated lymphocytes (lymphoblasts) (13). In contrast (Wiktor *et al.*, unpublished results), rabies virus is taken up by cultures of mouse macrophages, but does not seem to replicate in these cells and in mouse lymphocytes. In the present study, rabies virus did not repress the ability of unfractionated spleen cells to produce antibodies against sheep erythrocytes, whereas Newcastle Disease virus impairs the production of antibodies by rabbit spleen cells *in vitro* against bovine serum albumin (14).

The removal of the "adhering" cell elements from the mouse spleen suspension impaired the production of antibodies to rabies, and the addition of peritoneal-exudate cells restored it. One may surmise, therefore, that, similar to the results obtained with sheep erythrocytes as antigen (15), rabies antibody can only be produced *in vitro* by lymphocytes in the presence of an "accessory" cell. This "accessory" cell is probably a macrophage, unless other types of cells in the peritoneal cavity contributed to the restoration of the function of the immune system.

If macrophage interaction is the essential first step towards the production of rabies antibody, the better antigenicity of the preparation containing rabies virus adsorbed on sheep erythrocytes can not be explained by the greater aggregation of the virions on erythrocytes than in suspension since preparations containing rabies virions adsorbed on mouse erythrocytes did not initiate a better immune response *in vitro* than rabies virions maintained in suspension. The difference can not be explained by different amounts of virus available to produce the immune responses since, paradoxically, the amount of infectious virus was greater when rabies was maintained in suspension than when it was coated on sheep erythrocytes. This conclusion is further substantiated by the fact that an inactivated rabies vaccine also elicited an antibody response (Table 1).

If it is correct to assume that mouse peritoneal macrophages play an essential role in facilitating an immune response, then it is quite possible that a higher rate of phagocytosis of heterologous erythrocytes may provide a better carrier for rabies antigen for production of antibodies. This suggestion deserves further investigation.

The interaction between viral antigens and macrophages as an essential intermediary step in the production of antibodies against animal viruses may be dependent on intact virions being used as the antigens. It is possible that after dissociation of the virions, separate viral components may elicit antibodies in the absence of macrophages, and thus fall in the class of polymerized flagellin or solubilized material from sheep erythrocytes (16). The availability of such viral components after the dissociation of rabies virions (17) makes it feasible to undertake a study of their interaction with immune systems *in vitro*, and also to determine the role of various cell elements of the immune system involved in primary and secondary responses.

Finally, once an antibody against an animal virus has been produced in an *in vitro* system, a search should be instituted for other viral agents that, like the rabies virus, are capable of initiating an immune response *in vitro*.

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1. Fishman, M. J. (1961) *Expt. Med.* **114**, 837–856.
2. Notkins, A. L., Mergenhagen, S. E. & Howard, R. J. (1970) *Annu. Rev. Microbiol.* **24**, 525–538.
3. Sokol, F., Kuwert, E., Wiktor, T. J., Hummeler, K. & Koprowski, H. (1968) *J. Virol.* **2**, 836–849.
4. Wiktor, T. J., Sokol, F., Kuwert, E. & Koprowski, H. (1969) *Proc. Soc. Exp. Biol. Med.* **131**, 799–805.
5. Shortman, K., Williams, N., Jackson, H., Russell, P., Byrt, P. & Diener, E. (1971) *J. Cell Biol.* **48**, 566–579.
6. Marbrook, J. (1967) *Lancet* **ii**, 1279–1281.
7. Kissling, R. E. (1958) *Proc. Soc. Exp. Biol. Med.* **98**, 223–225.
8. Johnson, N. H. (1965) *Calif. Health* **23**, 35–39.
9. Macpherson, I. & Stoker, M. (1962) *Virology* **16**, 147–151.
10. Sedwick, W. D. & Wiktor, T. J. (1967) *J. Virol.* **1**, 1224–1226.
11. Wiktor, T. J., Kuwert, E. & Koprowski, H. (1968) *J. Immunol.* **101**, 1271–1282.
12. Cunningham, A. J. & Szenberg, A. (1968) *Immunology* **14**, 599–600.
13. Edelman, R. & Wheelock, E. F. (1966) *Science* **154**, 1053–1055.
14. Medzon, E. L. & Vas, S. I. (1964) *Can. J. Microbiol.* **10**, 535–541.
15. Shortman, K. D., Diener, E., Russell, P. & Amstrong, W. D. (1970) *J. Exp. Med.* **131**, 461–482.
16. Feldman, M. & Unanue, E. R. (1971) *Progress in Immunology*, First Intl. Congress of Immunology, ed. Amos, B. (Academic Press, New York), pp. 1379–1382.
17. Sokol, F., Stanček, D. & Koprowski, H. (1971) *J. Virol.* **7**, 241–249.