Immunogenicity of Concentrated and Purified Rabies Vaccine of Tissue Culture Origin* (33981)

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Although immunization of man against rabies was initiated in 1885 by Pasteur, there is still no completely satisfactory vaccine available for vaccination of human subjects. The availability of tissue culture systems for growth of rabies virus has made it possible to obtain virus preparations of considerably greater purity than those obtained from animal tissue. Nevertheless, such preparations, when used for production of antirabies vaccine, still contain large proportions of nonviral material and show only slightly improved immunogenicity (1, 2). The present report describes a method of purification and concentration of tissue culture-grown rabies virus which results in antirabies vaccine of highest known immunogenicity as determined in a mouse potency test.

Materials and Methods. Strains of rabies virus. The PM[1] strain of fixed rabies virus, obtained from the National Institutes of Health, was adapted to growth in WI-38 and propagated in these cells for 52 passages (3). A pool of virus was prepared in BHK21 cells (4) after purification by cloning using the plaque technique in agarose-suspended BHK-13S cells (5).

The Flury HEP strain (6) was adapted and propagated in WI-38 for 16 passages. A pool of virus was prepared in BHK21 cells after purification by cloning (see above).

CVS-26 was obtained from the National Institutes of Health. A pool of virus was prepared with 10% mouse brain suspension in 50% serum–water.

Tissue cultures. BHK cells and the WI-38 were propagated in 1-liter Blake bottles as described previously (7).

Infection of cells and virus propagation. Confluent monolayers of BHK cells and HDCS were infected with 2 ml of seed virus at an input multiplicity of about one pfu per cell in the presence of 50 μg/ml of diethylaminoethyl dextran (Pharmacia) (7). After adsorption for 1 hr the inoculum was removed, the cell monolayers washed once with warm PBS (8) and refed with Eagle's MEM (9) containing 0.3% BSA fraction V (Nutritional Biochemical Corporation) and double the amount (0.44%) of sodium bicarbonate. The infected cultures were incubated for 3 or 4 days at 32–33°, and the virus was harvested from the BHK cell cultures. Infected WI-38 cultures were trypsinized after 3-days' incubation and the infected cells mixed with freshly trypsinized noninfected cells in a ratio of 1:6. Aliquots of 2×10^7 cells from this mixture were planted in 1-liter Blake bottles in MEM containing 10% inactivated calf serum and incubated at 35° for 3 more days before harvesting the virus.

Concentration and purification of virus. Zinc acetate precipitation. The detailed method used for virus concentration and purification was described previously (10). Virus from infected tissue culture fluid was first

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[1] The following abbreviations are used throughout this paper: AV, antigen values; BPL, betapropiolactone; BSA, bovine serum albumin; CF, complement fixation; CVS, standard challenge virus; HA, hemagglutination; HAU, hemagglutination units; HDCS, human diploid cell strain; HEP, high egg passage; MEM, minimum essential medium; PBS, phosphate buffered saline; pfu, plaque-forming unit; PM, Pitman Moore.

precipitated by 0.02 *M* zinc acetate. The resulting sediment was resuspended in an ethylenediaminetetraacetate solution adjusted to pH 7.8 and passed through a Sephadex G-75 column. The effluent was treated as described with ribonuclease and deoxyribonuclease and sedimented for 60 min at 45,000*g*.

The pellet was resuspended in PBS without sonication and clarified by centrifugation at 1500 rpm for 20 min. The virus was concentrated 30- or 100-fold by this procedure.

High-speed centrifugation. BPL-treated infected tissue culture fluid (see below) was centrifuged for 90 min at 45,000*g* at 4°. The pellet was resuspended in PBS by sonic treatment for 60 sec at 10 kc (Raytheon ultrasonic oscillator) in a plastic tube.

Forced dialysis. BPL-treated tissue culture fluid, or the supernatant fluid obtained after the zinc precipitation, was dialyzed against Aquacide (Carboxymethylcellulose, Calbiochem, Los Angeles, California). The dialysis was carried out overnight at 4° and resulted in 20-fold concentration of the virus.

Inactivation of virus. Chemical. Virus preparations were mixed with BPL (Test Agar and Company, Inc., Detroit, Mich.) to a final concentration of 0.025% and maintained at 4° for varying lengths of time. The reaction was stopped by addition of sodium thiosulfite as described by Fayet *et al.* (11). Titrations of infectivity were carried out immediately after hydrolysis of the BPL.

UV irradiation. Virus preparations were exposed in petri dishes for different periods of time at a distance of 23 cm to radiation from a Westinghouse lamp type 782-L-20, which emitted radiation with an initial energy of 7 $\times 10^3$ ergs, cm^{-2} sec^{-1}. Samples were immediately diluted and titrated for infectivity. The depth of the fluid was either 0.15 or 0.30 mm.

Titration of virus infectivity. The infectivity of rabies virus in tissue culture fluids was determined by plaque assay technique in agarose-suspended BHK-13S cells as described previously (5, 12) and expressed as pfu/ml. In one experiment the infectivity was determined by intracerebral inoculation of 3-week-old mice with 0.03 ml/mouse and expressed as LD_{50}/ml.

CF test. The microtechnique described previously (10) was used for titration of CF activity of different vaccine preparations. Rabies immune serum was obtained from rabbits immunized with concentrated and partially purified viral antigen (PM strain) according to the method previously described (13). The reciprocal of the highest dilution of antigen showing partial reaction was taken as titer (CFU/ml).

HA test. Hemagglutinating activity of different virus preparations was determined according to the method previously described (14, 15). Goose erythrocytes at a concentration of 0.25% were used, and the test was carried out at 4° and pH 6.4. The reciprocal of the highest final dilution showing partial hemagglutination represented the number of HAU/ml.

Protein determination. Protein concentration was determined according to the method of Lowry *et al.* (16) with BSA as the standard.

Vaccine potency tests. The National Institutes of Health potency test was performed as described by Seligman (17) with a challenge dose of CVS-26 virus of 20–40 mouse LD_{50}. The reference vaccine used in this test was lot 173, supplied by the Division of Biological Standards, NIH. Because the method recommended for determination of the potency of brain tissue vaccine cannot be used for evaluation of tissue culture vaccines, the effective dose (ED_{50}) of reference and tissue culture vaccines was obtained by determining what dilution of the vaccine protected 50% of the mice vaccinated and subsequently challenged (18).

The AV were obtained by dividing the ED_{50} dilution value of the reference vaccine by that of the vaccine under the test. Vaccines are required by the NIH to have a minimum AV of 0.3 when tested against NIH lot 173 reference vaccine.

Results. Kinetics of inactivation of the virus. Because it was necessary in most experiments to inactivate rabies virus before immunization of mice, we investigated the kinetics of the inactivation process by either BPL or UV. Figure 1 shows that inactivation of rabies virus by 0.025% BPL follows an ex-

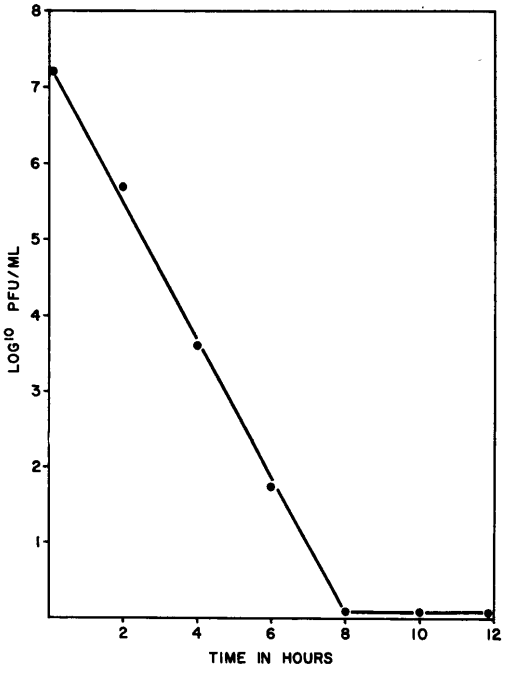

FIG. 1. Inactivation of rabies virus by BPL.

ponential pattern with respect to time of contact with the drug. After 8 hr at 4°, in presence of BPL, the concentration of the virus falls from $10^{7.2}$ pfu/ml to a nondetectable level. For safety, we inactivated the virus by maintaining the preparations in contact with BPL at 4° for 20 hr followed by 2 hr at 37° in order to hydrolyze BPL. Figure 2 shows an exponential relationship between time of exposure to UV irradiation and inactivation of rabies virus. Complete inactivation occurred within 2 or 4 min, depending on the depth of the infectious tissue culture fluid exposed to UV irradiation. As a protective measure, we irradiated for 10 min to 0.3-mm deep layer of infectious fluid.

Relationship between immunogenicity and CF activity of the vaccine. Attempts were made to correlate the immunogenicity in mice of the inactivated virus with its CF activity. HA activity could not be used for this purpose because the viral hemagglutinin was inactivated by BPL treatment. On the other hand, the CF activity of the virus was unaffected by BPL treatment or by UV irradiation. We have assayed 10 vaccine preparations by CF test and correlated the CF titers obtained with the protective power (ED_{50}) of the corresponding preparations. Results (Table I and Fig. 3) indicate that a close

TABLE I. Correlation between Complement Fixing and Immunogenic Activities of Inactivated Rabies Vaccines.

Vaccine lot no. (see also Table II)	CFU/ml (log 10)	ED_{50} (log 10)
1	0.90	1.67
2	1.81	2.54
3	0.60	1.42
4	2.51	3.22
5	0.90	1.74
6	2.51	2.92
7	0.60	1.23
8	2.11	2.53
11	0.90	1.70
12	2.11	2.84
Mean value	1.49	2.18

relationship exists between the CF and the protective activity of the inactivated virus. The average CF titers are about 5 times lower than the corresponding ED_{50} values obtained in the mouse protection test.

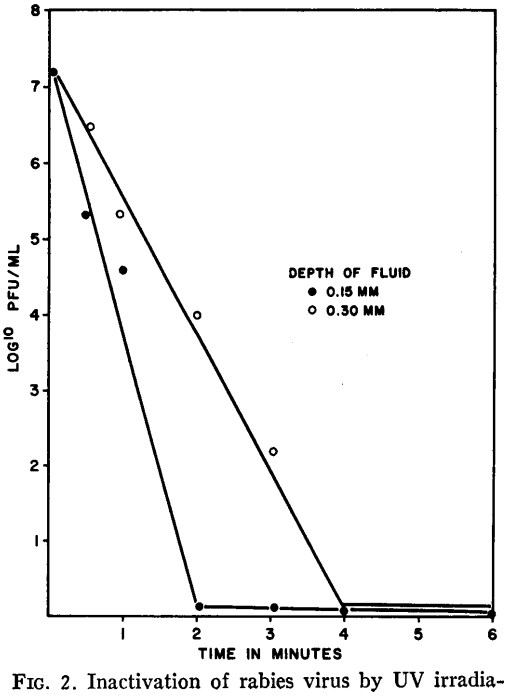

FIG. 2. Inactivation of rabies virus by UV irradiation.

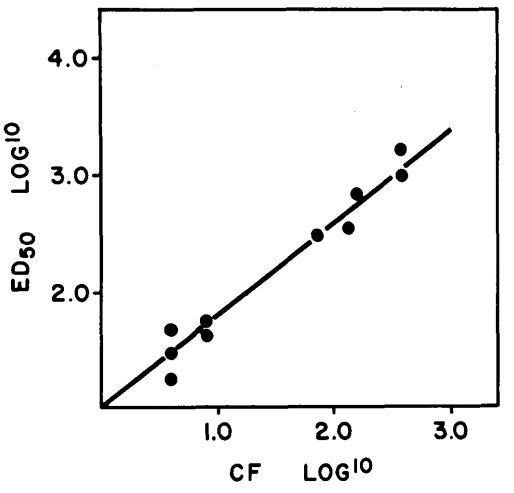

FIG. 3. Correlation between complement fixing and immunogenic activities of inactivated rabies vaccines.

Immunogenicity of rabies vaccine concentrated and purified by zinc acetate precipitation method. The results obtained in testing the immunogenicity of several concentrated and partially purified vaccines are shown in Table II. Except for one experiment with live HEP virus grown in BHK cells, all preparations were inactivated (after concentration and purification) by either BPL or UV.

As indicated by the recovery of infectivity and the decrease of protein content, the zinc acetate precipitation method produced a purified and highly concentrated virus. The high virus concentration resulted in markedly increased antigenicity of both live and inactivated vaccine preparations. The recovery rate of the CF activity was, however, lower than that of infectivity. The immunogenicity (AV) of the vaccine preparations varied considerably from experiment to experiment. The lowest recovery rate of AV was recorded for the live HEP-BHK and UV-inactivated PM-BHK preparations and the highest, for the same preparation of HEP-BHK virus, after inactivation by BPL.

Immunogenicity of rabies vaccines produced after concentration of the virus by other methods. These experiments were conducted with BHK tissue culture fluids infected with the PM strain of virus and treated with BPL prior to concentration. The same stock of virus was used in all the experiments. The antigenicity and immunogenicity of vaccines produced by various methods of concentration of virus indicate that the highest recovery rate of antigenicity and immunogenicity was obtained after concentration of the material by forced dialysis and the poorest recovery, after concentration by high-speed centrifugation only (Table III). The results obtained by zinc acetate precipitation method indicate that a considerable amount of CF antigen was retained in the supernatant fluid after removal of the precipitated virus.

Because the recovery rate of the infectivity in the zinc acetate precipitate and in the pellet after high-speed centrifugation is higher than that of AV (Table III), it seemed possible that not all antigenicity of the preparation is associated with the virus particle itself, but that the virion-free supernatant fluids may also contain rabies-specific antigens.

In order to investigate this problem further, a method of filtration of virus preparation through layers of membranes of various porosities developed by Strohmaier (19) was used[1] in an attempt to separate rabies virions from the soluble antigen. The BHK tissue culture fluids infected with the PM strain were subjected to the ultrafiltration procedures and the resulting preparations treated with BPL for inactivation of the virus. The results shown in Table IV indicate that the 40-fold virus concentration obtained after removal of the solvent by ultrafiltration through a membrane (Sartorius, Membranfilter GmbH, Göttingen, W. Germany) of 0.02–0.01 μ porosity resulted in almost total recovery of infectivity, HA and CF activity. Following inactivation by BPL, the preparation was tested for protective activity, and it was found that the increase in immunogenicity corresponded to the concentration factor. The filtrate was not infectious and

[1] Details of this study, conducted in collaboration with Drs. K. Strohmaier and L. Schneider from Bundesforschungsantalt für Viruskrankheiten der Tiere, Tübingen, Germany, will be published separately.

TABLE II. Immunogenicity of Rabies Vaccine Concentrated and Purified by the Zinc Acetate Precipitation Method.

Type of vaccine	Method of inactivation	Lot no.	Concentration factor	Infectivity before inactivation			Immunogenicity			CF activity	
				pfu/ml $\times 10^{-7}$	pfu/mg of protein $\times 10^{-7}$	Recovery rate (%)	AV	AV/mg of protein	Recovery rate (%)	CFU/ml	Recovery rate (%)
PM-BHK	BPL	1	×1	1.2	0.25	100	1.1	0.23	100	8	100
Expt. 1		2	×30	28.0	23.3	78	8.2	6.8	25	64	26
PM-BHK	BPL	3	×1	1.3	0.32	100	3.2	0.8	100	8	100
Expt. 2		4	×100	110.0	580	85	100.0	550	31	384	48
	UV	5	×1		See lot no. 3		3.4	0.85	100	8	100
		6	×100		See lot no. 4		51.0	270	15	384	48
PM-HDCS	BPL	7	×1	0.6	0.09	100	0.95	0.14	100	4	100
		8	×100	35.0	31.6	58	18.5	17.0	20	128	32
HEP-BHK	None	9	×1	6.0	1.7	100	10.0	2.9	100	8	100
		10	×30	120.0	1700	67	41.0	580	14	128	52
	BPL	11	×1		See lot no. 9		1.2	0.35	100	8	100
		12	×30		See lot no. 10		16.0	230	44	128	52

TABLE III. Immunogenicity of Concentrated and BPL-Inactivated Rabies Vaccine (PM Strain Propagated in BHK Cells); Comparison of Different Methods of Concentration.

Method of concentration	Concentration factor	CF activity		Immunogenicity	
		CFU/ml	Recovery rate (%)	AV/ml	Recovery rate (%)
None	×1	4	100	0.9	100
High-speed centrifugation	×100	128	32	13.5	15
Zinc acetate Sediment	×100	128	32	25.0	28
Supernatant	×20	32	40	6.7	37
Forced dialysis	×20	128	>100	≧20.0	≧100

was devoid of HA, CF, and protective activities.

In another experiment the original stock solution was freed from virus by ultrafiltration through a Sartorius membrane of 0.05 μ porosity and the resulting filtrate was concentrated 40-fold by ultrafiltration through a Sartorius membrane of 0.02–0.01 μ porosity. The concentrated preparation showed extremely high CF activity, no HA activity, and only traces of residual infectivity (Table IV). This "soluble antigen" preparation, after treatment with BPL, was tested for protective activity in mice and was found to be 8 times more effective than the original stock preparation containing both virus and "soluble antigen."

TABLE IV. Immunogenicity of BPL-Inactivated Rabies Vaccine (PM strain) Propagated in BHK Cells and Concentrated by Ultrafiltration Method.

Type of vaccine	Concentration factor		Infectivity $LD_{50} \times 10^{-6}$	HAU	CFU	AV after BPL inactivation
Starting tissue culture fluid	×1	Activity per ml	1.6	8	32	1.02
Concentrated virions and soluble antigen	×40	Activity per ml	100	256	1024	45
		Recovery rate (%)	>100	85	97	100
Concentrated soluble antigen alone	×40	Activity per ml	0.0016	0	1024	8
		Recovery rate (%)	0.005	0	97	21

Summary and Conclusions. It was confirmed in this study that tissue culture fluid harvested from rabies virus infected cell culture and inactivated by BPL or UV irradiation can be used for immunization against rabies. The protective activity of such preparation in mice was, however, generally not much higher than that of the NIH reference vaccine. The protective activity of this vaccine can be increased considerably by different methods of concentration of virions. The resulting preparations which showed also a high degree of purification, were up to 100 times more immunogenic than the reference vaccine in a mouse potency test.

It was demonstrated also that virions can be removed from rabies-infected tissue culture fluid, either by precipitation with zinc acetate, ultrafiltration, or by high-speed centrifugation. After concentration, such virion-free preparations showed a high CF and good immunogenic activity, but no HA activity. Further studies of such "soluble antigen" preparations may lead ultimately to the production of a highly purified and innocuous rabies vaccine which would be suitable for immunization of man.

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Chronic Aerogenic Tuberculosis in Mice* (33982)

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The intravenous injection of large numbers of virulent tubercle bacilli into mice usually results in death in 3 weeks or less, due to pulmonary insufficiency caused by tubercle formation and consolidation of the lung tissue. Mayer and Salamandra (1) reported that treatment of intravenously infected mice with isoniazid for 3 weeks prevented early death in these animals. The mice survived with chronic disease, and deaths occurred only after the seventieth day.

For the study of chronic mouse tuberculosis, it has been most useful to employ massive intravenous infection and treat with antituberculosis drugs which diminish the number of organisms, or to use attenuated virulent strains. Another approach is that of Gray and Mattinson (2) who infected mice by the intranasal instillation of fully pathogenic tubercle bacilli. By this technic Gray and Cheers (3) were able to define more clearly the "steady state," in which the number of viable units of tubercle bacilli remained constant for a long period of time. They proposed the term "immunological complaisance" for this phenomenon. In their experiments, 250 viable units of tubercle bacilli were introduced into the lungs of mice, which, considering the size of these lungs, seems a relatively large number.

The aerogenic infection route lends itself to controlled infection by small numbers of tubercle bacilli. We found that large numbers of mice may be infected simultaneously with approximately the same number of bacilli per mouse. The present report describes the natural history of chronic tuberculosis in mice infected by tubercle bacilli introduced in small numbers by this route.

Materials and Methods. The apparatus and the preparation of the single cell suspension used in aerogenic exposure of the mice to tubercle bacilli have been described elsewhere (4). The H37Rv strain, obtained from the Trudeau Institute, Inc., was subcultured in albumin Tween 80 liquid medium. Single cell suspensions were prepared and 1:100 dilutions were stored at —80° until needed. At

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