A Tissue Culture Color Test for Measuring Influenza Virus and Antibody.* (25118)

CHARLES R. GAUSH AND J. S. YOUNGNER
Dept. of Microbiology, School of Medicine, University of Pittsburgh, Pittsburgh, Pa.

The effect of poliovirus on metabolic activity of cultures of human embryonic tissue was observed by Enders, *et al.*(1) who found that infected cultures were less active than uninoculated controls in regard to acid formation. Later, they observed characteristic cytopathology in roller tube cultures and adopted this method for determination of the presence of virus since inhibition of acid formation was not always consistent(2). Salk, Youngner and Ward(3) developed a colorimetric test for assay of poliovirus and antibody *in vitro* using color change of phenol red as an indication of presence of virus or antibody. The present report describes a similar color test for titration of influenza virus and antibody. This test offers certain advantages over the more cumbersome egg technics currently in use.

Materials and methods. Media. Lactalbumin medium with 5% bovine serum was used for growing cells in 32 ounce prescription bottles and medium 199 without serum was employed for virus titrations and neutralization tests. Optimum color contrast could be obtained after 7 days if undiluted 199 was used with 2.5 ml of 5% sodium bicarbonate solution/100 ml of medium. Penicillin and streptomycin were added to concentration of 200 units and 200 μg/ml, respectively. Cell suspensions were prepared from kidneys of normal rhesus monkeys by methods previously described(4). Secondary cell suspension was prepared from bottle cultures by dispersing cells with 1:5000 sodium versenate and resuspending them in medium 199 without serum to concentration of 280,000 cells/ml. To each Wassermann tube was added 0.25 ml of cell suspension, final volume brought to 0.75 ml with medium 199 and the fluids overlaid with 0.5 ml of heavy mineral oil. *Virus pools* were prepared in monkey kidney roller tube cultures and stored at 4°C for a period not exceeding 2 weeks when they were replaced with fresh pools. *Immune sera.* Emulsified mineral oil adjuvant vaccines were prepared using Arlacel A as emulsifying agent. Antigens consisted of monkey kidney tissue culture pools or allantoic fluid pools of types A, A′, B, B′, and Asian influenza viruses. Normal rhesus monkeys or White Leghorn chickens were inoculated with adjuvant vaccines and were exsanguinated 6-8 weeks later following several additional aqueous vaccine inoculations. All sera were heated at 56°C for 30 minutes to destroy heat-labile non-specific inhibitors. Monkey sera were also treated with 8 mg trypsin (Difco 1:250)/ml of serum and chicken sera with 2 volumes 0.01 M potassium meta-periodate/volume of serum. *Hemagglutination and hemagglutination-inhibition* tests were performed according to standard methods(5).

Results. Virus titrations. Serial 10-fold dilutions of virus were made in medium 199 without serum and titrations performed as described elsewhere(3). Eight to 10 replicate tubes/dilution were used. Results of a typical titration are presented in Table I. In addition, virus titers were compared by color test and roller tube methods and, as observed in Table II, the mean color test titer was 0.56 logs lower than titers obtained in roller tubes.

Antibody titrations. Serial 2-fold dilutions of serum were made and to each dilution an equal volume of virus suspension containing about 400 $TCID_{50}$ of virus/ml was added. The mixtures were incubated at room temper-

TABLE I. Color Test Titration of Influenza Virus Infectivity Strain 575 MK-11 (Type A′).

Virus dilution	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Score*	8/8	8/8	8/8	8/8	6/8	2/8	0/8

Virus titer: $10^{5.5}$ ID_{50}/0.5 ml

* Numerator = No. of tubes infected (medium red). Denominator = No. of tubes inoculated.

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TABLE II. Comparison of Roller Tube and Color Test Infectivity Titrations of Influenza Viruses.

Virus	Type	Neg. log of ID_{50}/0.5 ml: Roller tubes	Color test	Diff. (RT − CT)
PR8 MK 12	A	6.0	4.8	1.20
" 10	A	4.74	4.11	.63
			4.0	.74
			4.0	.74
			4.5	.24
14	A	5.5	4.95	.55
575 MK 11	A′	5.5	5.4	.10
1210 MK 8	B′	5.5	5.0	.50
1210 E 4	B′	7.5	6.28	1.22
Peter MK 6	B′	3.25	3.56	−.31
				$\bar{X}$ = .56 logs

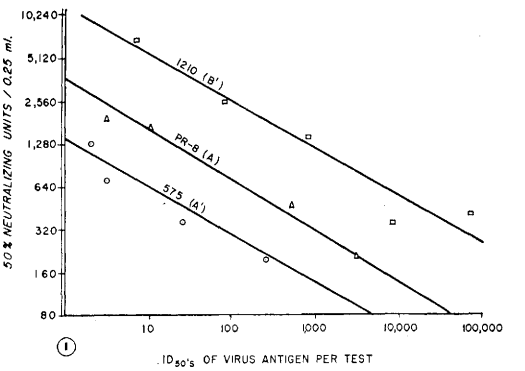
ature for 30 minutes and 0.5 ml of each serum-virus mixture was transferred to 3 or 4 replicate tubes. Each tube then received 0.25 ml of previously standardized cell suspension. Appropriate controls were incorporated as described previously(3). In an occasional uninfected tube, the pH did not fall to 6.6 or 6.8. However, it was observed microscopically that cultures having pH 7.4 or lower rarely displayed cytopathology. These tubes were considered negative (yellow) while tubes with pH higher than 7.4 were considered positive (red). A representative antibody titration is shown in Table III.

Influence of antigen concentration on antibody titer. Neutralization tests were performed with tissue culture and egg adapted strains in the color test and *in ovo* by varying both serum and virus concentrations. Types A, A′, and B′ were tested and, as shown in Fig. 1, a straight line relation existed: a 2-fold change in antibody titer resulted from each 10-fold change in antigen concentration. Slopes of neutralization curves did not differ significantly with virus type or when neutralized mixtures were assayed in the color test or *in ovo*. The curves in Fig. 1 are from combined data of egg and color test assays. Clarke and Tyrrell(6) assayed neutralized mixtures of influenza virus and antibody in monkey kidney roller tube cultures and in embryonated eggs and found the slope for the neutralization curve in cell culture was essentially the same as reported here. In contrast, a less steep slope was reported using *in ovo* assays than we observed.

TABLE III. Color Test Titration of Influenza Antibody Content of Immune Monkey Sera.

Antigen: Strain 575 (A′), 100 ID_{50}/tube			
Serum dilution	Immune sera: Anti-A′	Anti-A	Anti-B
1:20	0/3*	3/3	3/3
1:40	"	"	"
1:80	"	"	"
1:160	"	"	"
1:320	"	"	"
1:640	3/3	"	"
1:1280	"	"	"
1:2560	"	"	"
50% neutralizing units/0.25 ml serum	480	0	0

* Numerator = No. of tubes infected (medium red). Denominator = No. of tubes inoculated.



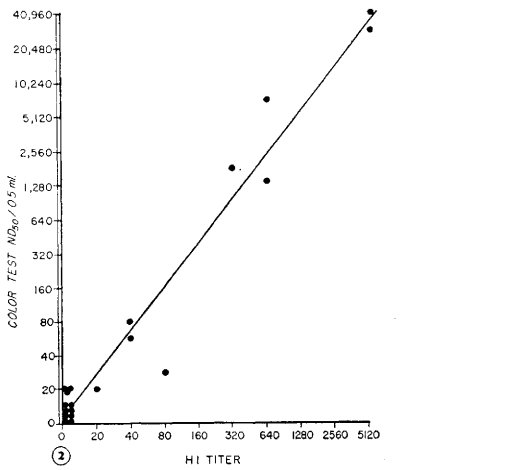


FIG. 1. The influence of amount of antigen on neutralizing antibody titers of influenza antisera.

FIG. 2. Correlation of color test neutralization and HI titers of periodate treated hyperimmune chicken sera.

Determination of antibody in immune

TABLE IV. Specificity of Hyperimmune Monkey Sera Tested with Homologous and Heterologous Virus Antigens.

Sera	Virus strain						
Antigen (immunization)	Swine	PR8 A	575 A′	Cup A′	FM-1 A′	Wright A′	1210 B′
Pre-vaccination	0	0	0	0	0	0	0
Post-vaccination							
Swine	60	0	0	0	0	0	0
PR8 A	0	90	0	0	10	10	0
575 A′	0	15	960	1920	1920	1920	0
"	0	10	60	30	480	30	0
"	0	15	60	30	480	30	0
1210 B′	0	0	0	0	10	0	1920
"	0	0	0	0	0	0	960
"	0	0	0	0	0	0	1920

All sera were heated at 56° for 30 min. and treated with 8 mg trypsin/ml serum. Serum antibody titers of all pre-vaccination sera were less than 1:10, lowest dilution tested. Values shown are 50% neutralizing doses/0.5 ml.

TABLE V. Color Test Titrations of Human Acute and Convalescent Sera Using Different Types of Influenza Virus as Antigens.

		Virus strain						
Patient	Bleeding	Swine	PR8 A	575 A′	Cup A′	FM-1 A′	Wright A′	1210 B′
Els	Acute	0	260	0	0	60	60	160
"	Conval.*	0	480	0	120	480	480	240
Sch	A	0	260	0	0	80	480	960
"	C	80	480	0	120	480	960	960
Swt	A	120	0	0	0	0	40	240
"	C	480	120	0	120	480	750	240
Wei	A	0	120	0	0	80	60	0
"	C	0	480	60	480	480	2980	0
Har	A	0	60	0	0	0	0	60
"	C	0	480	0	0	0	40	60

* Convalescent.

Sera were heated at 56° for 30 min. and treated with 8 mg trypsin/ml serum. Lowest dilution tested was 1:40 and values shown are 50% neutralizing doses/0.5 ml.

monkey sera. Cross neutralizations were performed with immune monkey sera using types A, A′. B′ and Swine as virus antigens. All pre-vaccination sera were free of neutralizing activity (Table IV). With immune sera, significant levels of clearly type specific antibody were obtained with the exception of some inhibition of PR8 virus with the A′ sera.

Determination of antibody in human sera. Acute and convalescent sera† from patients with clinically diagnosed influenza were tested with 4 types of virus to detect any significant increases in antibody level. Convalescent sera were obtained 2 weeks after the acute phase bleeding. Four-fold or greater rises in antibody titers were found with A′ virus antigens and especially with strains FM-1 and Wright (Table V). The response of Patient Har was significant with type A virus. Since the initial dilution of serum in these titrations was 1:40, the apparent increases of some titers may not be significant.

Hemagglutination-inhibition and color test antibody determinations with immune chicken sera. Cross neutralizations were performed using 5 type antigens and 4 periodate treated immune sera. Both HI and color tests were done with the same virus pools and the same series of serum dilutions. Antibody levels found with both methods were type specific and were increased with homologous type antigens (Table VI). The mean antibody titer by the color test method using homologous

† Kindly supplied by Elva Minuse, Virus Laboratory, School of Public Health, University of Michigan.

TABLE VI. Comparison of Color Test and HI Titrations of Hyperimmune Chicken Sera Using Homologous Influenza Virus Antigens.

	Antisera‡							
	PR8		PR301		Formosa		GL 1739	
Virus	CT*	HI†	CT	HI	CT	HI	CT	HI
PR8	30,700	5120	20	0	0	0	0	0
PR301	30	80	40,960	5120	60	40	20	20
Formosa	0	0	0	0	7680	640	0	0
GL 1739	80	40	20	0	20	0	1500	640
MB	0	0	0	0	0	0	1920	320

* No. of ND_{50}/0.5 ml. † Reciprocal of highest dilution showing inhibition. ‡ Heated at 56° for 30 min., treated with 0.01 M potassium periodate.

viruses and sera was 16.552 50% neutralization doses/0.5 ml while mean heterologous titer was 16 ND_{50}/0.5 ml. The mean homologous HI titer was 2368 compared to 12 for the heterologous titer. A plot of the titers is shown in Fig. 2 with regression line determined by method of least squares. The significance of linearity of regression was tested by means of analysis of variance. It was concluded that at the 0.10% confidence level there is a significant linear relationship over and above any curvilinear factors.

Conclusions. As with poliovirus, color test titrations of influenza virus and antibody represent an efficient, economic and accurate research tool which could be of great value in large scale epidemiological surveys and in detailed antigenic analyses of influenza virus strains. Economy of method can be demonstrated for about 18,000 Wassermann tube cultures, using secondary cells, can be prepared from kidneys of one 5 lb rhesus monkey. The methods currently employed for quantitative neutralization of influenza viruses are often time-consuming and cumbersome whereas the color test is unique in its simplicity and adaptability. The data presented also demonstrate high degree of serum specificity associated with this method and indicate that sensitivity of color test for detection of unneutralized virus is comparable to that observed *in ovo*.

Summary. 1. A tissue culture color test has been developed for titration of influenza virus and antibody using secondary monkey kidney cell cultures. 2. Virus titrations with the color test are comparable in accuracy with those done in roller tube cultures, mean titer being 0.56 logs lower in the color test. 3. Antibody content of immune sera (animal or human) can readily be determined and slopes of neutralization curves determined by color test are comparable to those found *in ovo*. 4. Color test neutralization titers and HI titers of the same sera show a significant association.

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