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Problems in the Detection of Rubella Virus in African Green Monkey Kidney Tissue Culture (32990)

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The isolation and study of viral agents has been dependent on innovation and new techniques. As these new methods have come into common usage, numerous difficulties have become apparent and more guide-lines and controls are required to insure the proper interpretation of results. In studies with rubella virus, the interference technique demonstrating the presence of rubella virus is in common usage. Recent studies using this technique with Coxsackie A-9 as a challenge virus have given false negative results in our laboratory. Samples previously showing high titer in AGMK demonstrate reduced titer or no detectable virus when re-titrated in different lots of this same tissue. The present study was undertaken to determine the relative sensitivity of various lots of African green monkey kidney tissue culture. At the same time an attempt was made to correlate the sensitivity of the tissue to rubella virus with complement-fixation titers for SV_5 of the donor monkeys and hemadsorption in the tissue culture system.

Materials and Methods. Virus strains. The RV strain (1) rubella virus in the fourteenth and sixteenth passage in AGMK was used to test the sensitivity of AGMK tissue culture used. Coxsackie A-9 was used as the challenge virus in the interference test.

Cell cultures for titrations. Primary African green monkey (*Ceropithecus aethiops*) kidney (AGMK) roller tube cultures grown in the presence of SV_5 hyperimmune rabbit antiserum were obtained from Microbiological Associates, Inc., Bethesda, Maryland and Flow Laboratories, Rockville, Maryland. They were maintained with 1.5 ml of a medium consisting of Eagle's basal medium Earle's balanced salt solution, 2% fetal bovine serum, 5 μg of amphotericin B, 10 μg of polymyxin, 30 μg of erythromycin, and 100 μg of streptomycin/ml.

Interference titration procedure. Infectivity titers of rubella virus in AGMK tissue cultures were determined by a modification of the enterovirus interference technique as described by Sever *et al.* (1). Briefly, serial 10-fold dilutions of virus samples were made in Hanks' balanced salt solution containing 2% fetal bovine serum, 100 μg of streptomycin, and 100 μg of neomycin/ml. Three tubes per dilution were inoculated with 0.2 ml of virus and incubated at 37°C. Eight to 10 days after inoculation, the medium was removed and replaced with fresh maintenance medium containing 100–1000 $TCID_{50}$ of Coxsackie A-9. The cultures were incubated for an additional 3–4 days and examined microscopically for the cytopathogenic effect of Coxsackie virus. The absence of cytopathogenic effect was interpreted as indicating the interfering effect of rubella virus. Fifty percent interference ($TCInD_{50}$) end points were calculated by the method of Spearman and Kaerber (2).

Complement-fixation procedure. The monkeys were bled when they were received and the sera were used in the complement-fixation test for SV_5 according to the microtechnique previously described (3).

Hemadsorption procedure. The hemadsorption test (HAd) was performed on the first 10 lots listed in Table II after the cells were held

TABLE I. Variations in the Sensitivity of African Green Monkey Kidney to RV P-14 Rubella Virus.

TC lot no.	Rubella $TCInD_{50}$/ml
1	<1.2
2	<1.2
3	2.2
4	2.9
5	3.5
6	3.7
7	2.9
8	≥ 3.5
9	3.5
10	2.7
11	3.7
12	1.7
13	1.7
14	3.7
15	3.7
16	<1.2
17	3.9
18	3.9
19	3.9
20	3.7
21	3.2
22	≥ 4.7
23	3.9

at 37° C for 21 days. The remaining lots were tested at the time the tissue was received (approximately 10 days after the cells were planted). The HAd test was a modification of the procedure described by Canchula *et al.* (4). Briefly, the cell sheet was washed once with Difco Bacto HA buffer, and 1 ml of 0.4% guinea pig red blood cells in the same buffer was added to each of 10 tubes of AGMK tissue culture. The tubes were incubated at 4°C for 30–45 min, and the RBC were removed from the cell sheet and washed once with 1 ml cold HA buffer. The tubes were examined immediately both macroscopically and microscopically for hemadsorption.

Results. Twenty-three lots of AGMK tissue culture received between May 31 and September 22, 1967 were tested for sensitivity with a single pool of RV P-14. The results presented in Table I show that three lots yielded titers of <1.2 $\log_{10}$ per ml, 2 lots gave titers of only 1.7, and one lot yielded 2.2 $\log_{10}$ per ml. The average titer for the remaining 17 lots was 3.6 $\log_{10}$ per ml. Seventy-four lots of AGMK received between June 29 and November 2 were tested for their sensitivity to a single pool of RV P-16 and the results are shown in Table II. Thirteen lots were reported as <1.8, <2.7, or <3.7 $\log_{10}$ per ml. The remaining 60 lots gave an average titer of 5.0 $\log_{10}$ of virus per ml. Table III summarizes the results of Tables I and II, showing the percentage of lots with varying degrees of sensitivity to rubella virus.

Of the 74 lots of AGMK tissue included in Table II, only lots 50–74 were prepared from monkeys tested for antibody to SV_5. Seven animals had CF titers of 1:4 to $\geq$ 1:64; one of these monkeys yielded tissue culture which was insensitive to rubella. The two lots of insensitive tissue were derived from monkeys with $<$1:4 CF titers to SV_5. The maintenance medium contained SV_5 hyperimmune serum. Hull (personal communication) has found that SV_5 infection of the donor monkeys has no influence upon the sensitivity of the tissue culture prepared from these animals.

Four of the 74 lots of AGMK tissue culture tested were positive for hemadsorbing agent or agents. Of these four lots, three were considered insensitive to rubella virus. The results are shown in Table II.

Discussion. The data clearly demonstrates that there is a wide variation in sensitivity in African green monkey kidney tissue culture to infection with rubella virus. Although duplicate titrations in the same tissue will give slightly different titers, the variation is normally less than 0.5 log/ml. In Table II, two instances of tissue prepared from the two kidneys of the same animal gave the same titer for each lot of tissue as seen by lots 40 and 41 and the second pair, 42 and 43. The tissue used for the studies was obtained from two commercial sources, and both sources prepared tissue with a great variation in sensitivity to rubella virus. This variation in sensitivity has been found not only in our laboratory, but has been reported from several laboratories throughout this country and England which are engaged in work on rubella virus (personal communications). Variation in sensitivity of AGMK to rubella virus is of importance in viral isolation studies, both for epidemiological purposes and for the

TABLE II. Correlation of African Monkey Kidney Sensitivity to RV P-16 with Hemadsorption Results and Monkey SV_5 Complement Fixation Titers.

TC lot no.	Rubella $TCInD_{50}$/ml	HAd	TC lot no.	Rubella $TCInD_{50}$/ml	HAd	SV_5 CF titer for monkey
1	<3.7	—	38	5.5	—	
2	4.7	—	39	5.2	—	
3	3.5	—	40	6.2	—	
4	3.5	—	41	6.2	—	
5	<3.7	—	42	5.2	—	
6	<3.7	—	43	5.2	—	
7	3.5	—	44	5.5	—	
8	<3.7	—	45	5.5	—	
9	<3.7	—	46	4.5	—	
10	<3.7	—	47	4.5	—	
11	5.7	—	48	5.2	—	
12	3.7	—	49	6.2	—	<1:4
13	3.5	—	50	4.5	—	<1:4
14	<3.7	—	51	4.9	—	<1:4
15	5.7	—	52	4.5	+	1:8
16	5.2	—	53	3.7	+	1:8
17	5.5	—	54	5.5	—	≥1:4
18	4.9	—	55	6.0	—	<1:4
19	4.9	—	56	5.5	—	<1:4
20	<3.7	—	57	4.2	—	<1:4
21	<3.7	—	58	5.7	—	≥1:64
22	5.4	—	59	5.5	—	1:4
23	4.5	—	60	5.9	—	
24	3.5	—	61	5.7	—	<1:4
25	<2.7	+	62	5.9	—	<1:4
26	4.9	—	63	<2.7	+	<1:4
27	4.2	—	64	4.9	—	<1:4
28	4.2	—	65	5.2	—	1:4
29	5.7	—	66	4.0	—	<1:4
30	4.7	—	67	5.0	—	<1:4
31	5.5	—	68	5.0	—	<1:4
32	<2.7	—	69	<1.8	—	<1:8
33	5.9	—	70	3.9	—	<1:4
34	3.8	—	71	5.2	—	<1:4
35	6.2	—	72	6.2	—	ND
36	5.0	—	73	5.7	—	ND
37	5.5	—	74	5.5	—	1:16

isolation of rubella virus which may be shed from the throats of individuals who have received attenuated live rubella vaccine in field trials. If insensitive AGMK tissue is used for such isolation studies, the presence of rubella virus may not be detected. Under these circumstances, it would be possible to evaluate a potential vaccine as one which elicits high immunogenic reponse without the shed of the virus from the vaccinated individual. The use of insensitive tissue, however, may have prevented the detection of the virus in clinical specimens. It is possible that wide acceptance of a vaccine evaluated in such tissue could result in undue risk to the general public.

The sensitivity of AGMK tissue to rubella virus is also important in the determination of the levels of live attenuated virus found in tissues of experimental animals sacrificed receiving a live attenuated virus (5). One of the markers of attenuation has been limited tissue distribution of minute amounts of virus in vaccinated animals as compared to the spread

TABLE III. Degree of Sensitivity of AGMK Tissue Culture to Rubella Virus.

$TCInD_{50}$/ml (log_{10})	Rubella virus seed			
	RV P-14		RV P-16	
<1.2	3/23	13.0%		
1.0–1.9	2/23	8.7%	1/74	1.4%
2.0–2.9	4/23	17.4%		
<2.7			3/74	4.1%
<3.7			9/74	12.2%
3.0–3.9	13/23	56.5%	9/74	12.2%
4.0–4.9	1/23	4.3%	16/74	21.6%
5.0–5.9			30/74	40.5%
6.0–6.9			6/74	8.1%

[a] 9/23, 39.1% considered to be insensitive.
[b] 22/73, 30.1% considered to be insensitive.

of rubella virus throughout the entire body of test animals inoculated with wild strains of the virus. In attempts to isolate virus from the tissues or in the search for virus shed from the throat, the presence of minute amounts of attenuated virus may be highly significant. Every effort should be made, therefore, to insure that the results of such laboratory tests are accurate.

The sensitivity of AGMK to rubella virus is also important in the determination of the amount of attenuated virus included in a vaccine pool for the vaccination of human subjects. Unless adequate standard rubella virus titrations are included as controls in the titrations of vaccine pools, the vaccine may include more, or in some instances less, virus than was intended, and thus may produce less or greater degrees of immunity than would normally be expected. To compare lots of vaccine or strains of rubella virus used in the preparation of vaccines, it is important that the amount of virus used be accurately determined. The comparison of vaccines prepared from different attenuated strains of rubella virus is a difficult task. Unfortunately, the possiblity that they may be of different strengths due to inaccurate titration in insensitive tissue adds to the difficulty of making an accurate comparison.

The results shown in Tables I and II indicate that some lots of tissue require 10–100 and even 1000 $TCInD_{50}$ doses of rubella virus to give any indication of infection. Such variation in sensitivity results in added difficulty in the determination of neutralization titers of immune sera since this test is dependent on the ability of an immune serum to neutralize 0.5 log_{10} of rubella virus (1). In our laboratory, the use of such lots of AGMK tissue culture has led to the inability to detect the small amounts of virus All such tests for the detection of small quantities of rubella virus are closely related to the sensitivity of the AGMK tissue culture system. It is, therefore, important that each lot of tissue be tested with a known rubella virus standard to determine its sensitivity to the particular strain of virus used in any one laboratory. It is not sufficient to include a rubella standard at one dilution. The standard must be included as a titration. Only in this way can the true sensitivity of the tissue be detected. Assuming that the rubella standard used for these studies had been inoculated into AGMK tubes as the control in the undiluted state, the wide variation in sensitivity would have gone unnoticed. Of the lots tested with RV P-16, approximately 18% were insensitive to 1000 $TCInD_{50}$ doses.

After one has become aware that a problem exists, it becomes necessary to determine the cause of the problem and to take corrective steps, or to determine how to avert the problem by other procedures. The determination of the cause of the recent insensitivity of AGMK tissue may prove insurmountable at this time, however, data will be collected in an effort to determine the cause or causes for this variation in sensitivity to rubella virus. In an effort to determine the presence of cytopathogenic agents or hemadsorbing agents in the kidney tissue, 10 lots were held for 21 days and observed for CPE and tested for hemadsorption agents. Of the lots tested, no evidence of CPE or hemadsorbing agents was detected after 21 days incubation. Although no agents were detected in these 10 lots by this procedure, it does not preclude their presence since no subcultures were made. Numerous viruses have been shown to be present in simian tissue culture and have been described by Hull *et al.* (6) and Hsiung (7). Up to 30% of monkey kidney cultures

may show hemadsorbing agents after incubation of several weeks (8).

Since it would be of considerable advantage to know of the resistance of a lot of tissue prior to use, all lots of AGMK received are routinely tested for hemadsorption agents the day the tissue arrives and prior to its use in the laboratory. This practice has shown four lots to contain hemadsorbing agents. Of these four lots, two were found to be completely insensitive to rubella virus, a third lot showed reduced titer. A fourth lot yielded results indicating no appreciable decrease in sensitivity to rubella virus. Although the data is limited, it appears that there would be a 3 to 4 chance of those lots of tissue which exhibit hemadsorption to also show resistance or insensitivity to rubella virus. During the period in which the hemadsorption test has been used, only one lot, no. 69, has been highly resistant to rubella virus and yet negative by the hemadsorption test. Two lots, nos. 34 and 70, have shown reduced sensitivity to rubella virus and were also HAd negative. Since the interference test is time-consuming, it would appear to be worthwhile to refuse to use hemadsorption-positive lots of AGMK tissue culture for studies with rubella virus.

For lack of a better method for determining resistance of AGMK tissue culture, the HAd method may possibly offer a way of detecting some insensitive lots of tissue prior to use. Although hemadsorbing agents may not be the only cause of resistance to rubella virus, it would appear that in some instances an agent or agents which cause hemadsorption also induce resistance to rubella virus.

Summary. The sensitivity to rubella virus of 96 lots of AGMK tissue culture was evaluated using the enterovirus interference technique. Wide variation in sensitivity was found leading to the classification of 32 of the lots tested as resistant to rubella virus. The use of these resistant lots of tissue culture for the isolation or titration of rubella virus would lead to invalid test results. Appropriate controls are needed to assure the validity of studies in which rubella virus is detected with African green monkey kidney tissue culture.

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