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Immunohemagglutination Test for Rapid Detection and Assay of Rubella Antibodies.* (32111)

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Teratogenicity and widespread incidence have given rubella considerable contemporary importance. *In vitro,* rubella virus renders a number of mammalian cells resistant to superinfection with cytolytic viruses (8,15,17,19), produces recognizable cytopathology in others (6,18,19,21), but has lacked hemagglutinating or hemadsorbing activity(9). Apart from the fluorescent antibody technique described by Brown *et al*(1), slower, less sensitive complement fixation and neutralization tests have been the only procedures available for detection and assay of rubella antibodies(8,14,16, 20,21). This report describes the use of tanned chicken red blood cells coated with rubella virus for qualitative and quantitative determination of rubella antibodies in animal and human sera.

Materials and methods. Tissue culture. Cultures of LLC-MK_2 cells were grown and maintained in medium 199 containing 1% horse serum. BHK_{21} cells of the WI_2 clone, kindly supplied by Dr. S. A. Plotkin, were grown as monolayer cultures in Eagle's basal medium containing 10% calf serum, 10% tryptose phosphate broth (Difco) and antibiotics (penicillin, streptomycin and amphotericin B)(18). For cell maintenance and virus growth, the serum concentration was reduced to 2-5% and the tryptose phosphate broth omitted. For the growing of BHK_{21} cells in suspension, Eagle's basal medium, containing a double concentration of amino acids and vitamins, 10% calf serum, and 10% tryptose phosphate broth was used. For cell maintenance and virus growth in suspension culture, the double strength Eagle's basal medium was used with 5% fetal calf serum as the only supplement. All suspension cultures contained 3 ml of 4% methylcellulose (1500 cp) per 100 ml of medium. Monolayers of RK_{13} cells were grown and maintained in medium 199, 15% fetal calf serum and antibiotics(6). Uninfected tissue culture fluid (TCF) was passed in parallel with virus and used for controls.

To grow virus in monolayer cultures, cells were infected with undiluted seed virus, allowed one hour at room temperature for adsorption, then maintenance medium added and bottles reincubated at 35°C. Virus was grown in suspension cultures by infecting cell suspensions at high multiplicity with the viral inoculum, and the cultures agitated on a rotary shaker at approximately 300 rpm. Both virus and control TCF were harvested by

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freezing and thawing and the crude fluids were centrifuged at 2000 rpm for 20 minutes. The supernate was stored as small aliquots in sealed ampoules at —70°C.

Virus was titered in stoppered BHK_{21} tube cultures which were read for cytopathology 4-6 days after inoculation. The pH of the tube cultures was adjusted by adding 0.1 ml of sterile 1.4% $NaHCO_3$ solution to each tube on the second day after infection. Usually no further adjustments were needed before final reading.

Virus used to inject guinea pigs was grown in LLC-MK_2 cells and had a titer of 10^3 $TCID_{50}$/ml, while rabbits were injected with virus grown in BHK_{21} cells to a titer of $10^{6.5}$ $TCID_{50}$/ml. Tanned erythrocytes were sensitized with the latter batch of virus except in a few tests where the HPV-77 (obtained *via* Dr. G. C. Brown from Dr. P. D. Parkman) strain of rubella virus (RV) was used after several passages in RK_{13} cells.

Serum samples. Young albino guinea pigs, weighing 250-500 g, and rabbits, weighing 2-2.5 kg, were used to prepare rubella immune sera. Animals were caged individually, bled by intracardiac puncture and then injected intraperitoneally with 1 ml of undiluted virus. Injections were repeated 2 times at 10-day intervals and blood collected 2 weeks after the third and last injection. Acute and convalescent human sera from cases of rubella, selected by clinical appearance, confirmed by fluorescent antibody assay(1), kindly made available by Dr. G. C. Brown, were included. All sera were inactivated by heating at 56°C for 20 minutes before testing.

Test procedure. Blood from white Leghorn chickens was drawn by heart puncture and collected in Alsever's solution. Erythrocytes were separated by centrifuging, washed 3 times with buffered saline solution (BSS) and diluted to 10% in BSS. The erythrocytes suspension was further diluted to 2.5%, incubated for 10 minutes at 37°C with an equal volume of freshly prepared 1:120,000 tannic acid (Merck), washed once, the supernate poured off, and sufficient BSS added to restore the concentration to 2.5%. One volume of 2.5% tanned cell suspension and one volume of virus suspension were added to four volumes of 0.15 M-pH 6.4 phosphate buffer and incubated at room temperature for 10 minutes. The sensitized, tanned cells were then centrifuged at 1000 rpm for 5 minutes, washed twice with 1:100 normal rabbit serum in saline (NRS) and diluted with that solution to give a final 1% cell suspension(13). The test was carried out by making serial twofold dilutions of the heat-inacitvated sera in 1% NRS. To each tube or panel cup an equal volume of sensitized tanned red cells was added and mixed. Readings were made after a minimum incubation at room temperature of one hour (usually 1-3). Controls consisted of sensitized tanned red cells mixed with NRS or tanned red cells sensitized with normal TCF. A positive agglutination pattern appeared as a uniformly thin layer of red cells covering the bottom of the container whereas the negative pattern appeared as a round button or doughnut of settled cells. When test sera or NRS diluent was not inactivated, hemagglutination patterns were not seen and the red cells hemolyzed. The highest serum dilution giving a positive pattern was considered the titration endpoint.

Results. None of the virus-injected animals had rash or showed other pathologic signs during the period of observation.

Animal sera. Table I presents data on the antibody response of guinea pigs and rabbits, as determined by the immunohemagglutination test, after administration of rubella virus. It is seen that all guinea pigs have responded with the production of antibodies as indicated by significant rises in HA titer, when pre- and post-viral serum samples were compared. Pre-immunization sera had titers less than 1:2, except in one case, while post-immunization sera showed titers ranging from 1:16 to 1:512. This Table also shows the HA response of rabbits to injection of rubella virus grown in BHK_{21} cells using erythrocytes sensitized with virus grown in the same cell line. In all 3 rabbits pre-injection sera showed titers less than 1:2 while post-injection showed significant rises using both viral and control fluids. However, a difference, 4-fold or greater, between viral *vs* TCF titers was found in each case. To avoid possible nonspecific TCF positive reactions in the sera of guinea pigs

TABLE I. Reciprocal HA Titers of Animals Injected with Rubella Virus.*

Animal	Red Cell Sensitizing Antigen†	Serum Titer Before Virus	Serum Titer After Virus
Guinea pig No.			
165	V	<2	> 64
	TCF	<2	4
167	V	<2	> 16
	TCF	<2	2
168	V	4	>512
	TCF	<2	64
169	V	<2	>512
	TCF	<2	64
170	V	<2	>512
	TCF	<2	64
Rabbit No.			
1	V	<2	2048
	TCF	<2	512
2	V	<2	64
	TCF	<2	16
3	V	<2	512
	TCF	<2	32

* Guinea pigs injected with LLC-MK_2-grown virus; erythrocytes sensitized with BHK_{21}-grown virus. Rabbits injected with BHK_{21}-grown viruses; erythrocytes sensitized with same lot of virus, or with BHK_{21} normal TCF.

† V, rubella virus; TCF, tissue culture fluid.

they were immunized with LLC-MK_2-grown virus while the tanned erythrocytes were sensitized with viral antigen or TCF prepared in BHK_{21} cells.

Human sera. Two groups of human sera were obtained, one set in acute-convalescent pairs from clinically diagnosed rubella cases, and another set supplied and assayed as coded samples. Parallel HA and FA titers obtained with these sera are presented in Table II. Acute sera showed lower titers than convalescent in all but 2 cases where, by immunohemagglutination test, members of each pair were equal. In the majority of cases antibody titers measured by both methods were equal or similar. However, in the paired serum series there was no clear pattern to the instances of difference.

Effect of antigen specificity and concentration. Since the aforementioned experiments involved the WM strain of rubella virus, it was of interest to employ a different virus strain. Therefore, a human serum sample, convalescent from clinically and serologically diagnosed rubella, was titrated for antibody content using two strains of rubella, WM and HPV-77, with the results presented in Table III. By the fluorescent antibody technique, the serum had an antibody titer of 1:128 and it is seen that, compared with control values, both viral strains gave much higher titers. Since it was noticed that viral preparations having different infectivity titers

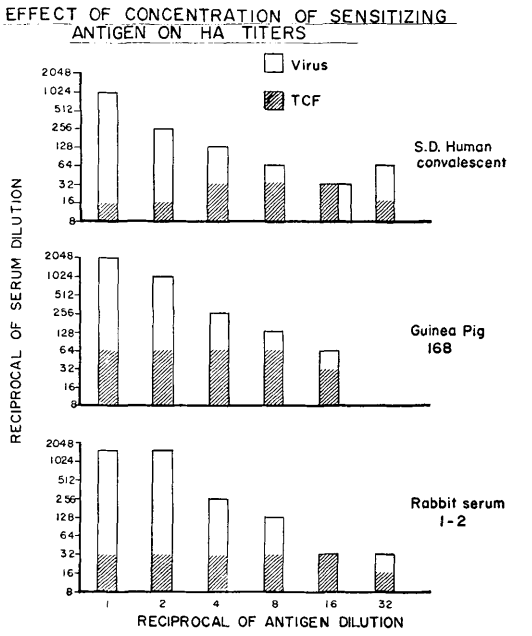

FIG. 1.

TABLE II. Reciprocal of HA and FA Titers of Rubella Antibodies in Human Sera.

Patient		Serum No.	Serum Titer HA	FA*
Gc	a	1	8	8
	c	2	32	64
BB	a	3	2	<8
	c	4	8	8
AD	a	5	2	<8
	c	6	8	64
SV	a	7	32	<8
	c	8	32	256
SD	a	9	16	16
	c	10	128	128
RJ	a	11	32	<8
	c	12	32	32
HM	a	13	ND	ND
	c	14	64	<8
Coded Sera		15	<2	<8
		16	<2	<8
		17	<2	>16
		18	<2	<8
		19	16	>16
		20	16	16
		21	<2	8-16
		22	<2	<8
		23	16	32
		24	<2	<8
		25	<2	>64
		26	<2	<8

* Starting serum dilution is 1:8, thus, <8 equivalent to negative serum.
ND = not done. a = acute serum; c = convalescent serum.

TABLE III. HA Titers in Human Convalescent Serum Using Different Strains of Rubella Virus.

Red Cell-sensitizing Antigen	Infectivity Titer ($TCID_{50}$/ml)	Reciprocal of HA Serum Titer
WM-BHK_{21}	$10^{6.5}$	512
BHK_{21}-TCF	0	16
HPV-77, RK_{13}	10^{4}	128
RK_{13}-TCF	0	32

Antigens used diluted 1:2 and test run in plastic panels.

tended to yield differing HA titers for the same serum sample, an experiment of the influence of concentration of sensitizing antigen was conducted. WM rubbella virus, $10^{6.5}$ $TCID_{50}$/ml, grown in BHK_{21} cells, and the corresponding TCF control were diluted in buffered saline and the antigen dilutions incubated with tanned erythrocytes were incubated at room temperature with serial dilutions of known immune rabbit, guinea pig, and convalescent human sera. Results illustrated in Fig. 1 show the relationship between antigen dilution and immunohemagglutination titer. These data show that immunohemagglutination titers with TCF-sensitized cells did not change appreciably with dilution while titers with virus-sensitized cells were markedly higher when the amount of viral antigen was increased.

Discussion. Neutralization *in vitro* of the cytopathogenic(6,18,21), interfering(8,9) or immunofluorescing(1) properties of the virus and complement fixation(16,20), until recently, have been the techniques available for detecting antibodies to rubella virus. Based on the unusually low antibody titers obtained when tested under conditions standard for other viruses, rubella infectivity neutralization seems to lack sensitivity, while interference tests are cumbersome and time-con-

suming. Although rubella complement fixation tests have proved specific(14,16), limited sensitivity is also suggested by the low titers found in the serum of convalescents. Moreover, preparation of rubella complement-fixing antigen is lengthy and expensive, with the final product frequently showing anticomplementary activity(16). The fluorescent antibody technique, though rapid and sensitive, has yet to find more acceptance even in the presence of requisite equipment and trained personnel. Indirect hemagglutination tests have been described for the detection of antibodies to mumps and Newcastle disease (2), adeno-(3,10), herpes simplex(13), and polioviruses(7). The same technique, using sheep or rabbit erythrocytes, has been reported as unsuccessful for rubella virus by Parkman *et al*(9) and by Veronelli (Studies of Rubella, dissertation for degree of Doctor of Philosophy, Univ. of Michigan, 1966). In the preliminary experiments of this study and using rubella virus grown to a titer of 10^3 $TCID_{50}$ per ml in LLC-MK_2 cells, no significant hemagglutination titers were seen using sera of hyperimmunized animals. A few sera, however, showed low titers, not distinctly different from those with TCF-sensitized red cells. With higher titered antigen, subsequently available by growing rubella virus in BHK_{21} cells(18), higher and more dependable hemagglutination titers have been obtained. Dependence of serum hemagglutination titers on the potency of the viral antigen used to sensitize the red cells is evident from data in Fig. 1 and may explain earlier experience with this technique. The lack of success in earlier attempts using concentrated and pellet-purified virus preparations may be due to the fact that the major viral antigen responsible for the hemagglutination of red cells when mixed with specific antibodies is separable from the infectious virus particle as was reported by Ross and Ginsberg with adenovirus passive hemagglutination(11). The importance of antigenic potency is exemplified by serum sample No. 10 which gave hemagglutination and fluorescent antibody titers of 128, but a hemagglutination titer of 1024 (Fig. 1) with an 8-fold increase in concentration of the sensitizing antigen. The data in Table II also show a lower threshold of detection of antibodies by the immunohemagglutination method.

Detection of rubella antibodies is of obvious importance. Applications include determination in the obstetric patient for an index of susceptibility to disease, standardization and estimation of the potential protective value of human gamma globulin preparations, and diagnosis of disease in order to select or evaluate control methods, such as vaccination or antiviral drugs. Provided that high titered rubella virus is used to sensitize tanned erythrocytes, with the immunohemagglutination method described here, serum antibody may be titrated more easily and to levels higher than most other available methods and detected at lower concentrations.

Summary. Tanned adult chicken erythrocytes were found to adsorb rubella virus and there to be agglutinated by rubella antibody. Pre- and postimmunization sera were collected from guinea pigs and rabbits given live WM strain rubella virus grown in LLC-MK_2 or BHK_{21} cells. Paired sera from human cases of rubella were also tested. In animals preimmunization titers were <2 and ranged from 64 to 2,048 postimmunization. Titers in convalescent humans ranged from 8 to 128. Significantly lesser increases in titer were seen with erythrocytes sensitized with uninfected tissue culture medium. Comparison of human hemagglutination (HA) and fluorescent antibody titers showed good agreement. HA titers varied with the infectivity titer of the sensitizing RV and HPV-77 strain RV reacted similarly.

Addendum. While this manuscript was in preparation, Meyer and Parkman have reported hemagglutination activity of rubella virus using red cells of one-day-old chicks. (International Conference on Vaccines Against Viral and Rickettsial Diseases of Man Pan American Health Organization, WHO, Washington, D. C. Nov., 1966.)

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Immunofluorescent Studies of Virus-Induced Rabbit Papilloma (Shope) *in vitro*.* (32112)

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Virus-cell relationship in Shope virus-induced papillomas of rabbit skin has attracted the particular interest of investigators in the tumor virus field in respect to the "masking" of this virus in papillomas of domestic rabbits (1).

Recently Noyes and Mellors(2) have carried out immunofluorescent studies on the tissue sections of the neoplasia and have shown that the viral antigen could be clearly demonstrated in papillomas of cottontail rabbits while it was detectable but less conspicuous in the sections of domestic rabbit papillomas. On the other hand, it has been reported that papillomas of both cottontail and domestic rabbits can yield tumorigenic nucleic acid by phenolic extraction although the level of activity of the extracts from the latter tissue is much lower than that from the cottontails (3,4). These findings, however, were all obtained at the tissue level and little study has been done on the system at the cellular level.

The present article deals with immunofluorescent studies of cells derived from Shope virus-induced papillomas cultured *in vitro*, especially from a point of view of possible persistence of the virus-related antigenic substance(s) in these cells.

Materials and methods. Virus. Glycerinated papillomatous tissue of Kansas cottontail rabbits was employed as the source of Shope papilloma virus. A 10% aqueous extract was prepared and used as virus preparation. The virus was inoculated on to the skin of domestic rabbits by scarification method(4). Papillomatous growth appeared at the site of inoculation after 2 to 3 weeks.

Cell culture and media. A small piece of papillomatous tissue was removed from the skin of the animal and finely minced with

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