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Rubella Hemagglutinin Prepared with Alkaline Extraction of Virus Grown in Suspension Culture of BHK-21 Cells. (32038)

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The preparation of a new type of alkaline extracted potent complement fixing (CF) antigen without serum components(3) suggested the possibility that this antigen might contain hemagglutinin activity. The report of Meyer(4) of agglutination of erythrocytes from one-day-old chicks by a rubella antigen prompted us to test our antigen with the same type of cell.

The present report describes the specific hemagglutination (HA) of young chicken erythrocytes with alkaline extracted rubella antigens prepared from infected BHK-21/13S cells grown in suspension culture. Factors which enhance both the preparation and testing of the antigen and the performance of the hemagglutination inhibition (HI) test are also indicated.

Materials and methods. Virus strain and cell culture techniques have been described previously(3). Aliquots of the seed virus (8th passage level in this laboratory) were stored at -70°C . The titer of this virus was about 10^7 TCID₅₀/ml. Fifteen ml of seed virus was inoculated into 800 ml of suspension culture with about 10^6 cells/ml. The infected cells were harvested on the 7th day.

In the HA and HI test, antigen and serum

diluent was 0.4% bovine-albumin-borate-saline solution and erythrocyte diluents were 0.15 M NaCl-phosphate buffers with indicated pH(7). The blood of chickens at indicated ages was drawn from the heart using a 5 ml syringe containing 3 ml of Alsever's solution(6). From each of 10 chickens 0.5 ml to 1.5 ml of blood was collected, pooled and stored in Alsever's solution. A 10% stock suspension was prepared in Alsever's solution and stored up to a week at $+4^{\circ}\text{C}$. The 0.25% working suspension was prepared daily in erythrocyte diluent. Serum specimens for HI tests were treated with kaolin and chicken red cells according to the arbovirus technique (7) with minor modifications: the specimens were diluted 1:5 with borate saline, pH 9.0 (0.2 ml of serum and 0.8 ml of borate saline), in a 15 ml conical centrifuge tube. An equal volume (1.0 ml) of 25% kaolin suspension (prepared in borate saline, pH 9.0) was added; the tubes were shaken vigorously 2 to 3 times during 20 minutes incubation at room temperature and centrifuged in the same temperature at 2,500 RPM for 30 minutes (International Centrifuge, Model PR-2, Rotor No. 269[†]). Without collecting the supernate,

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[†] Use of trade names is for identification only and does not constitute endorsement by the Public Health Service of the U. S. Dept. of HEW.

TABLE I. A Representative Rubella Hemagglutinin Titration at Various pH Levels Using 0.25% Red Cells of 2-Day-Old Chickens.

Antigen lot	pH*	Antigen dilution								Cell control
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	
A	6.0	+	+	+	+	±	—	—	—	—
	6.2	+	+	+	+	+	—	—	—	—
	6.4	+	+	+	+	±	±	—	—	—
	6.6	+	+	+	+	—	—	—	—	—
	6.8	+	+	+	+	±	—	—	—	—
	7.0	+	+	+	±	—	—	—	—	—
	7.2	+	+	±	—	—	—	—	—	—
	7.4	+	+	±	—	—	—	—	—	—
B DGV,† pH	7.0	+	+	+	+	+	—	—	—	—
	5.8	+	+	+	+	+	+	+	—	—
	6.0	+	+	+	+	+	+	—	—	—
	6.2	+	+	+	+	+	+	+	—	—
	6.6	+	+	+	+	+	+	—	—	—
	6.8	+	+	+	+	+	±	—	—	—
	7.0	+	+	+	+	+	—	—	—	—
	7.2	+	+	+	+	—	—	—	—	—
	7.4	+	+	+	±	—	—	—	—	—
	7.6	+	+	+	—	—	—	—	—	—
	7.8	—	—	—	—	—	—	—	—	—
	8.0	—	—	—	—	—	—	—	—	—

* This is the pH of the final reaction mixture.

† Antigen was diluted also in dextrose-gelatin-veronal buffer(7). All the other antigens were diluted in 0.4% bovine-albumin-borate-saline solution (BABS).

0.1 ml of a 50% chicken red cell suspension (red cells of both young and adult chickens were used) was added to each tube and then incubated in an ice water slurry for 30 minutes with shaking at 10-minute intervals. The mixture was centrifuged at 2,000 RPM for 10 minutes in the cold, and the clear supernate, now a 1:10 dilution of serum, was removed.

Disposable microtiter U-plates were used in all hemagglutinin and serum titrations. The volume of antigen dilutions used for titrations was 0.05 ml, and an equal volume of 0.25% erythrocyte suspension was added to each well while the plates were agitated by a mechanical reciprocating vibrator. Particular attention was taken to keep all reagents for titration in an ice water slurry and to place the plates, immediately after sealing with tape, on the ice water slurry to ensure cold temperature during the development of the HA pattern. After 1-1/2 hours incubation the hemagglutination was read according to the influenza virus HA technique(1). The end point of titration was taken as the highest dilution of antigen causing a plus-minus degree of agglutination, or as the dilution interpolated between a plus and negative.

For the HI test, the reagent volume of serum and antigen dilutions was 0.025 ml. The antigen was diluted to contain 4 hemagglutinating units per 0.025 ml. After preincubation of the serum-antigen mixture for various times and temperatures, the reagents were cooled on the ice water slurry and 0.05 ml of erythrocyte suspension was added to each well. The test was incubated and read as indicated above. The highest initial serum dilution causing complete inhibition of agglutination was considered as the antibody titer. A separate control titration of the diluted antigen was carried out with each HI test using 0.025 ml volumes to determine the actual reactive units of antigen which went into the test. Each HI test also included the following: 1) serum controls to detect non-specific hemagglutinins, 2) erythrocyte-diluent controls, and 3) known positive serum.

Results. Effect of pH on hemagglutination. The hemagglutinin titers were higher at the acid pH levels as shown in Table I. This pH dependence varied slightly from one lot of antigen to another, but the antigen titers were always 2 to 8 times higher at pH 5.8 to 6.6 than at pH 7.0 to 7.2. This was found with all types of antigens tested, such as non-

sonicated, sonicated and tween-ether extracted antigens.

Since the antigens were alkaline (pH 9), it was necessary to select a diluent for the 0.25% erythrocyte suspension which would change the pH of the reaction mixture to 6.2. This pH of 6.2 was chosen somewhat arbitrarily from the optimal range and thus was used as the final pH for the mixtures of antigen and red cells(7).

Effect of age of chickens on sensitivity of erythrocytes in hemagglutination. Blood samples were collected from young chicks from 2 different groups. In 2 experiments the use of erythrocytes from 2- and 3-day-old chickens resulted in the same or slightly higher antigen titers than those obtained with one-day-old chickens. Cells from 7- and 11-day-old chicks gave significantly lower antigen titers. There was no significant HA with adult chicken erythrocytes at pH 6.2. Thus, the erythrocytes of 2-day-old chicks were used in all the later experiments.

Effect of storage of red cells on hemagglutination. Several pools of a 10% erythrocyte suspension in Alsever's solution were stored for 2 weeks at +4°C without losing their sensitivity for HA. After 5 days storage the cells began to hemolyze; therefore, they were centrifuged and resuspended in fresh Alsever's solution before use in the test.

Formalinized red cells(2) of 2-day-old chickens could be used after several weeks storage at +4°C. However the hemagglutinin titers were much lower with formalin treated cells than with fresh cells.

Effect of temperature on hemagglutination. Hemagglutination could be demonstrated at +4°C; but at room temperature (about 22°C) only a trace of HA was observed, and at 36°C no HA was detected. Before mixture, the reagents were brought to the temperature at which the test was conducted. Once the HA pattern developed at +4°C, it remained stable for at least 24 hours at room temperature.

Factors influencing alkaline extraction of hemagglutinin from infected cells. Simultaneous extractions of rubella hemagglutinin from infected cells with 0.1 M glycine-NaOH buffer pH 9.0, and the same buffer containing 0.4%

bovine albumin showed that the hemagglutinin titers were at least two times higher with added bovine albumin. This effect became apparent only when the extraction was carried out for 5 hours or longer at 35°C. At the end of each extraction time the cells were sonicated for 30 minutes and then centrifuged. The hemagglutinin titers increased progressively during the first 30 hours extraction and thereafter no further increase was detected (Table II).

The infected cells were not disrupted even after prolonged alkaline extraction, but the disruption of cells by sonication before alkaline treatment failed to release the same amount of antigen. Maximal hemagglutinin titers were obtained after 30 to 60 minutes sonication. Tween-ether extraction(5) increased the hemagglutinin titers of non-sonicated antigens 4 to 16 times but it did not significantly increase the titer of sonicated antigen. The highest titers of antigen were between 1:1024 and 1:2048.

The hemagglutinin was routinely prepared by extraction for 30 hours in glycine-NaOH buffer containing 0.4% bovine-albumin, followed by 30 to 60 minutes sonication. The antigen was clarified by centrifugation for 30 minutes at 10,000 RPM in the Spinco.

Thermal stability of the alkaline extracted hemagglutinin. The alkaline extracted antigens were stored at +4°C and -70°C for several weeks and at 35°C overnight with no significant decrease in the titer. At 56°C the hemagglutinin was rapidly inactivated as shown in Fig. 1.

Factors influencing hemagglutination inhibition test. Fifteen serum specimens with varying levels of antibodies were tested after: 1) kaolin adsorption, 2) kaolin and red cell adsorption and 3) no adsorption. In a 1:10 dilution of some untreated sera, inhibitors of rubella hemagglutinin were present which were absorbed by kaolin treatment. Some of the untreated or kaolin absorbed specimens contained chicken red cell agglutinins in low titers. In later experiments all serum specimens were absorbed with both kaolin and red cells.

The effect of temperature and time of pre-incubation of the serum-antigen mixtures

TABLE II. Effect of Time of Alkaline Extraction at 35°C on Final Hemagglutinin Titer with Two Lots of Antigen.

Lot	Extraction time (hr)	Antigen dilution									
		1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
A	0	+	+	±	—	—	—	—	—	—	—
	1	+	+	±	—	—	—	—	—	—	—
	5	+	+	±	—	—	—	—	—	—	—
	10	+	+	+	+	+	±	—	—	—	—
	20	+	+	+	+	+	+	±	—	—	—
	30	+	+	+	+	+	+	+	±	—	—
	60	+	+	+	+	+	+	+	±	—	—
B	5	+	+	+	+	+	—	—	—	—	—
	30	+	+	+	+	+	+	+	+	±	—
	60	+	+	+	+	+	+	+	+	±	—

(before addition of erythrocytes) were evaluated at +4°C for 1 hour, 2 hours and overnight, and at 22°C and 37°C for 1 hour. After preincubation, the serum-antigen mixtures were cooled on an ice slurry before addition of erythrocytes. The antibody titers of all sera tested were somewhat lower with incubation at +4°C for 1 hour. There was some indication that the titers of a few sera were one tube higher with preincubation at 37°C for one hour and also at +4°C overnight, but this difference was not consistently observed. Unless otherwise indicated, HI tests were carried out with a preincubation period of 2 hours on an ice slurry. The daily control titration of the diluted antigen indicated that 4 units of antigen were usually present. The daily variation in titer of the positive control serum was not more than one dilution.

Appearance and persistence of hemagglutination inhibition antibodies. HI antibody titers in acute and convalescent specimens collected from young adults during a rubella epidemic are shown in Table III. HI antibody appearance correlated well with CF antibody obtained using alkaline extracted rubella antigens(3) and with neutralizing antibody. In the convalescent phase sera the HI titers were 1:80 to 1:640.

The persistence of HI antibodies in children several years after rubella infection is indicated in Table IV. The antibody titers were 1:40 to 1:320, 2 to 3 years after rubella infection. The persistence of HI antibodies was also demonstrated in a survey of serum specimens collected from females in the Atlanta area. In groups tested between the ages of 6-35 the HI test was positive in 80-90% of the individuals.

Discussion. Rubella hemagglutinating activity could not be demonstrated until sensitive erythrocytes were tested with high titered virus. Our results indicate that rubella hemagglutinin of high titer can be readily obtained from virus grown in suspension culture of BHK-21/13S cells. Alkaline extraction is not required for hemagglutinin preparation since it was not used by Meyer(4). However, this extraction technique permits fetal bovine serum to be used without kaolin treatment for the growth and maintenance of cells in suspension culture. In addition, treatment of infected cells prepared with alkaline buffer for periods of 30 hours greatly increases the yield of hemagglutinin. When using erythrocytes of 1- to 3-day-old chicks the optimal conditions

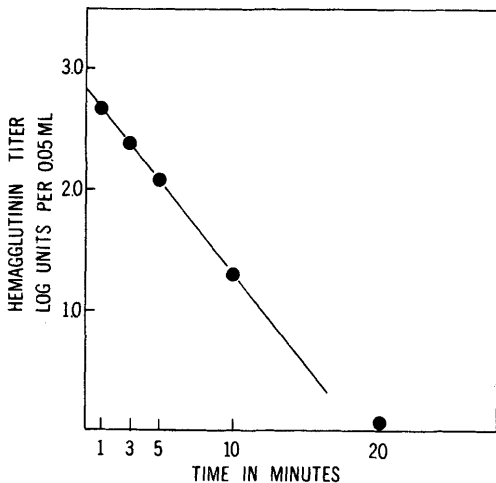


FIG. 1. Inactivation rate of alkaline extracted rubella hemagglutinin at 56°C.

TABLE III. Hemagglutination Inhibition (HI), Neutralizing (NT) and Complement-Fixing (CF) Antibody Titers in Serum Specimens Collected from Young Adults During a Rubella Epidemic.

Specimen No.		Days after onset of rash	Antibody titers		
			CF	NT	HI
493	S1	0	<4	<8	<10
	S2	22	128	64	≥640
497	S1	3	16	<8	<10
	S2	29	256	16	≥640
714	S1	1	<4	<8	<10
	S2	29	16	16	320
751	S1	0	<4	<8	10
	S2	22	128	64	160
755	S1	1	<4	<8	<10
	S2	22	128	16	160
771	S1	0	<4	<8	<10
	S2	21	64	8	320
772	S1	1	<4	<8	<10
	S2	23	128	16	320
776	S1	0	<4	<8	<10
	S2	25	256	32	160
778	S1	3	<4	<8	10
	S2	23	32	8	640
779	S1	2	16	<8	<10
	S2	22	128	16	80
780	S1	1	<4	<8	<10
	S2	22	64	8	160
781	S1	3	<4	<8	<10
	S2	25	32	16	160
862	S1	0	32	<8	<10
	S2	27	128	16	160
865	S1	2	8	<8	<10
	S2	30	128	8	160

for HA include low temperature and a pH of 6.2.

The alkaline extracted hemagglutinin was capable of detecting rubella HI antibodies. The optimal conditions for hemagglutination inhibition have not been defined. Such factors as the preincubation of serum-antigen mixtures at various times, temperatures and pH levels should be carefully evaluated as should different techniques for removing non-specific inhibitors from serum.

It is not the purpose of the present study to compare the sensitivity or specificity of this rubella HI technique with that of Meyer(4). With either method, HI has several advantages when compared with other rubella serologic techniques since it is an easily and rapidly performed method of detecting previous experience with rubella.

Initial studies have shown that significant HI antibodies develop after illness. This indicates the usefulness of the HI test in addition to the CF test in the diagnosis of rubella infection.

Summary. Rubella hemagglutinin of high titer was prepared by alkaline extraction of infected BHK-21/13S cells grown in suspension culture. After this treatment, sonication or tween-ether extraction increased hemagglutinin titer 4-16-fold. Optimal conditions for hemagglutinin titration using erythrocytes from 1- to 3-day-old chicks included a low temperature and a pH of 6.2. A limited number of stability tests indicated that alkaline extracted hemagglutinin was

TABLE IV. Persistence of Rubella HI Antibodies in Children* 1 to 3 Years After Infection.

Patient No.	Time of rubella infection	Time serum collected	HI antibody titer
845	June, 1964	Mar., 1964	<10
		Aug., 1964	160
		Feb., 1965	40
		Oct., 1965	40
864	June, 1961	Aug., 1966	40
		June, 1962	320
864	June, 1961	May, 1964	320
		June, 1962	<10
899	May, 1964	Jan., 1965	40
		Oct., 1965	40
		Jan., 1966	80
		Aug., 1966	40
2051	June, 1964	Dec., 1963	<10
		Oct., 1965	320
		Aug., 1966	640
2061	May, 1964	Jan., 1964	<10
		Aug., 1964	80
		Dec., 1964	80
		Oct., 1965	80
		Apr., 1966	160
2071	May, 1964	Aug., 1966	160
		May, 1964	<10
		June, 1964	320
		Oct., 1965	320
2081	May, 1964	Aug., 1966	160
		Aug., 1964	640
		Sept., 1964	320
		Dec., 1964	160
		Feb., 1965	80
2082	May, 1964	Oct., 1965	80
		Aug., 1964	80
		Feb., 1965	40
		Oct., 1965	40

* Sera obtained from a children's home in a respiratory illness study conducted by the Respiratory Unit, Virology Section, NCDC.

stable at -70°C and $+4^{\circ}\text{C}$. The hemagglutinin was used to detect rises in HI antibody in paired serum specimens. Hemagglutination inhibition antibodies were present in titers of 1:40 to 1:320, 2 to 3 years after rubella infection.

The supervision of all tissue culture preparation by Mrs. Anna Hall and the excellent technical assistance of Miss Marianne Davis and Miss Ludie Holland are gratefully acknowledged.

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Rubella Complement Fixing Antigen Prepared by Alkaline Extraction Of Virus Grown in Suspension Culture of BHK-21 Cells. (32039)

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Schmidt and Lennette(9) have adapted an alkaline extraction method for preparation of rubella complement fixing antigen using monolayer cultures of BHK-21 cells. A technique for the growth of high titered rubella virus in suspension culture of the same cells has recently been reported by Vaehri *et al* (13).

The present report describes the preparation of rubella complement fixing (CF) antigen with alkaline extraction of virus grown in suspension culture of BHK-21 cells. Also reported are the high levels of antibody and their duration, measurable with this type of rubella antigen.

Materials. Virus strains. The rubella virus strain RA 27/3(13) for CF antigen preparation was kindly supplied by Dr. Antti Vaehri, Wistar Institute of Anatomy and Biology, Philadelphia, Pa. When received, it had been passaged 4 times in WI-38 cells and

about 25-30 times in BHK-21/13S cells. In this laboratory 8 more passages were made in the latter cells.

The Gilchrist strain of rubella virus used for neutralization tests was obtained from Dr. J. L. Sever, National Institutes of Health, Bethesda, Md. Its previous history is unknown, but in this laboratory it was passaged in primary or continuous African green monkey kidney (GMK) cells. It was used in neutralization tests at the 10th and 11th passage level.

Cell cultures. Clone 13S of BHK-21 cells for suspension culture was supplied by Dr. Antti Vaehri and was maintained according to the technique reported(13) with minor modifications. The growth medium, called here Wistar growth, was Eagle's minimum essential medium prepared in Hanks' solution with a double concentration of amino acids (except 1 X glutamine) and vitamins, supplemented with 0.1 mg per liter of ferric nitrate, 4.5 g per liter of glucose (final concentration), 10% tryptose phosphate broth

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