A New Technique for Preparation of Rubella Complement Fixing Antigen.* (31011)

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A method for assay of rubella complement-fixing antibody, based on the use of cell-associated antigens has been reported by Sever *et al*(1) and by Stern(2). The antigens employed by these authors consist of the "packed cells" of infected tissue cultures subjected to 3 cycles of freezing and thawing, and homogenization.

The present paper reports on the alternative possibility of using antigenic material present in the supernatant fluid of rubella infected tissue cultures. The technique as developed permits the easy preparation of antigenic material in sufficient quantities for routine serological work. The finding that at least part of this activity is associated with a component smaller than the intact virus particles enhances the interest in the study.

Materials. 1. Virus. The WM strain, isolated in this laboratory in 1962(3) was employed. The strain was passed 5 times in LLC-MK 2 cells, 5 times in BS-C-1 cells, and 10 to 13 times in GMK-AH 1 cells. Uninfected culture fluid passed in parallel with the virus was used for control preparations.

2. Tissue culture. The GMK-AH 1 (continuous green monkey kidney) cell line was employed. The characteristics of growth of rubella virus in this cell line were reported by Günalp(4), through whose courtesy the line was obtained. Growth medium for the cells consisted of 40% Puck's N-16, 40% Eagle's Minimum Essential Medium (MEM), 10% fetal bovine serum, and 10% horse serum.

After inoculation the tissue cultures were maintained on media consisting of equal parts of N-16 and Eagle's MEM with variable amounts of sera. Some preparations were obtained using 2% horse serum, but this was later replaced by inactivated guinea pig serum† (GPS) at concentrations between 0 and 5%, generally 2%.

Methods. 1. Preparation of antigens. (See Fig. 1). *a. Procedure for inoculation and harvesting.* Monolayers grown in bottles with 170 cm^2 effective surface area (2.3×10^7 cells/bottle) were washed with 15 ml of Eagle's MEM and inoculated with approximately 2×10^6 Interfering $Doses_{50}$ in 10 ml. After 2-3 hours absorption at room temperature, the inoculum was replaced with 40 ml of maintenance medium with 2% GPS, followed by further addition of 10 ml of serum free medium at days 4 and 7. The supernatant fluid was harvested 10-11 days after inoculation and sodium azide added to a final concentration of 0.08%; the same concentration of azide was maintained in all solutions used for dilution or dialysis. The effects of variation of GPS concentration or time of harvesting on antigen yield have not been rigorously investigated; under the conditions outlined the cytopathogenic action of rubella virus was incipient at day 4 and marked at days 7 and 10.

b. Concentration and dialysis. Preparations were concentrated to 1/10 to 1/15 of the original volume of harvested fluid by forced dialysis against polyethylene glycol‡ for 10-12 hours. The procedure was carried out at 4°C and was preceded or followed (according to the volumes handled) by clarification through centrifugation at 2,500 rpm for 15

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† Guinea pig sera was obtained in our own laboratory or purchased from Grand Island Biological Co. Both were inactivated at 56°C for 30 minutes. When using the former the media was filtered to ensure sterility.

‡ Carbowax 4000. Union Carbide Co., South Charleston, West Va.

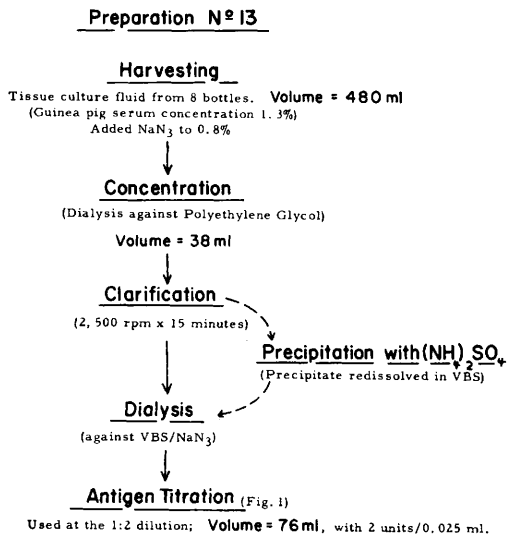

FIG. 1. Example of preparation of complement fixing antigen.

minutes in a refrigerated centrifuge. Finally the concentrates were dialyzed against veronal buffer saline (VBS) for 24 hours at 4°C with 3 changes of VBS.

c. Precipitation with ammonium sulfate. In some preparations partial purification was obtained by addition to the fluid concentrates (chilled in an ice bath) of an equal volume of saturated ammonium sulfate solution (kept at room temperature). The mixtures, in which a precipitate formed immediately upon addition of the salt, were kept for 6-8 hours in an ice bath and then centrifuged at 4,000-6,000 rpm at 4°C for 15 minutes. The precipitate, which floated as a creamy top band, contained the antigen and was easily redissolved in a small volume of chilled VBS. This step removed one-third of the protein content without altering the antigenic activity; the anticomplementary activity, however, increased slightly. In view of this result ammonium sulfate precipitation was employed only when the material was intended for experimental work on purification or characterization of the antigen.

d. Storage. Antigen preparations were stored at −65°C. No significant alteration in their antigenic or anti-complementary activities has been found due to storage over limited periods of time or to repeated freezing and thawing.

2. Technique for complement fixation assay. A modification of the microassay employed at Dr. Stuart-Harris' laboratory (Sheffield, England) was used(5). All reagents were dispensed in 0.025 ml drops on "Perspex" plates.

The hemolytic system consisted of sheep red cells at a final concentration of 0.4% and hemolysin (titer 1:16,000) at a final concentration of 1:1600. The test, like the original microtechnique of Fulton and Dumbell(6), was therefore carried out with maximally sensitized cells, which resulted in agglutination of the non-hemolyzed red cells. Guinea pig complement was stored in Richardson's solution(7) and titrated in the presence of antigen by incubation at 4°C for 12 hours and, after addition of the hemolytic system, for 2 hours at 37°C.

Titration of antigens was carried out with 2 sera with antibody titers of 1:64 and 1:48 which were selected as standards. Times and temperatures of incubation were the same as those employed in the antibody assay. The unit of antigen was defined as the highest dilution that results in a 50% (2+) or greater fixation with the highest dilution of serum. Fig. 2 gives an example of antigen titration; it should be noted that each dilution of antigen received a slightly different amount of complement based upon results of a previous titration. Once the potency of a given preparation is known and the proper dilution made, complement need be titrated only in its presence before each use. The antigen shown in Fig. 2 was employed in the 1:2 dilution in order to deliver 2 units of antigen per drop.

For the antibody assay 2.5 units of complement and 2-3 units of antigen were employed; fixation was carried out at 4°C for 16 hours and after addition of the hemolytic system the plates were incubated for 2 hours at 37°C. Endpoints were read at the highest dilution showing 50% (2+) or greater fixation of complement.

Results. 1. Specificity of the reaction and antibody titers. Several preparations of antigen, used in dilutions that provided 2-3 units per drop, gave essentially identical results when repeatedly tested with a group of acute and convalescent sera. Preparations using

Immune (Convalescent) Serum dilution	Antigen dilution (Preparation no. 13) 1:1	1:2	1:4	1:6	1:8	1:12	1:16	No Antigen	Control Antigen 1:2	No Antigen	Control Antigen 1:2	Antigen 1:2	Negative (Acute) Serum dilution
1:4	4	4	4	4	3	1	Trace	0	0	0	0	0	1:4
1:8	4	4	4	4	2	0	0	0	0	0	0	0	1:8
1:16	4	4	4	3	1-2	0	0	0	0	0	0	0	1:16
1:32	4	4	4	2	1	0	0	0	0	0	0	0	1:32
1:48	3	4	3	1	Trace	0	0	0	0	0	0	0	1:48
1:64	0	1-2	0	0	0	0	0	0	0	0	0	0	1:64
1:128	0	0	0	0	0	0	0	0	0	0	0	0	1:128
Dilution of compl. (2.5 u)	39	50	50	50	52	52	54	54	50	54	50	50	
Compl. 2.5 u	0	0	0	0	0	0	0	0	0	0	0	0	
Recheck 1.2 u	Trace	0	0	0	0	0	0	0	0	0	0	0	
0.6 u	3-4	2	2	2-3	2	2	3	2	2	2	2	2	

FIG. 2. Example of antigen titration. The complement re-check test was carried out by reacting a drop of each antigen dilution with the amount of complement used (2.5 u) and with a 1:2 and 1:4 dilution of the latter, in the absence of sera. 0 to 4+ stand for the degree of complement fixation (*i.e.*, complete to no hemolysis).

control tissue cultures had no antigenic activity.

The successive titers of sera from 6 patients with typical rubella are presented in Fig. 3. Five of these patients had titers of less than 1:4 at days 1 and 3-4 after onset of rash. The other patient had an early antibody rise (day 3). By day 25 all these patients had titers between 1:8 and 1:32. Peak titers were observed at 3-6 months and persisted with only slight decrease for at least a year after infection.

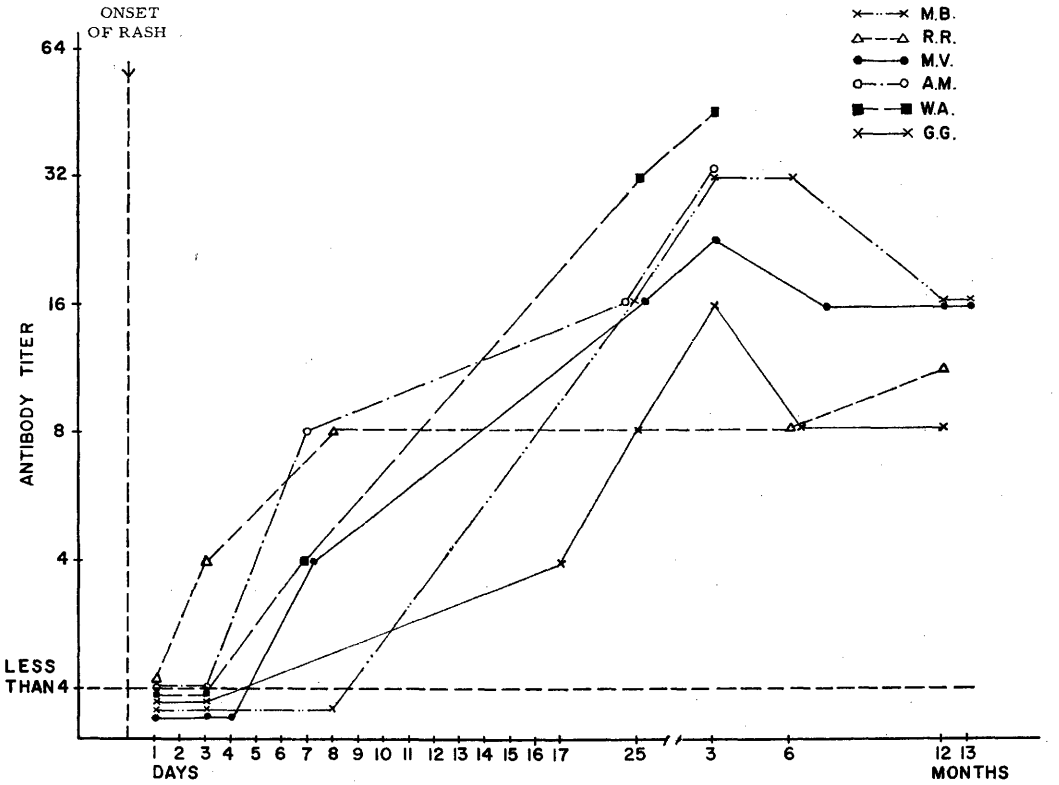

FIG. 3. Antibody titers in early convalescence and first year following rubella infection.

2. Further purification of the antigenic material. Chromatographic techniques were applied in an attempt to achieve a greater degree of purity. The sample consisted of tissue culture fluids, prepared with horse serum maintenance medium, concentrated and purified by low (2500 rpm × 15 minutes) and high (18,000 rpm × 120 minutes) speed centrifugation and ammonium sulfate precipitation. The sample, as is the case with all horse serum preparations, was too anticomplementary for use in the complement fixation test.

Filtration through a short column (25 × 2.5 cm) of Sephadex G-200, equilibrated with VBS, gave a single broad protein profile in the "eluate." Four fractions of this protein peak were tested, the first of which was highly anticomplementary. The second, which had the highest protein content, was relatively inactive, while the third fraction had the highest antigenic titer and little anticomplementary activity. The last fraction had little activity.

The antigenic material was adsorbed and eluted from diethylaminoethyl cellulose (DEAE) columns (25 × 2.5 cm). A continuous gradient of increasing NaCl osmolarity was applied for resolution and the antigenic activity eluted between 0.050 M and 0.152 M NaCl, with the peak at 0.108 M.

Aliquots of material fractionated on DEAE were filtered through 50 and 10 mμ millipore filters; the filtrates apparently contained most of the complement fixing antigen, although this was not thoroughly quantitated. This clearly indicates that the antigen is much smaller than the intact virus.

Discussion. The need for rapid serological tests for rubella has been repeatedly pointed out and has already resulted in the development of the indirect immunofluorescent assay (8) and a complement fixation test employing "packed-cells" antigens(1,2). The technique reported here has not yet been extensively compared with these or other methods of antibody assay but the characteristics of antigens derived from infected tissue culture fluids and the methods employed in their preparation can be briefly discussed.

Essential to the method outlined for antigen preparation are the use of GPS in the maintenance medium, the harvest of the fluid phase of the cultures, with the exclusion of all cells and the concentration of this material.

The use of inactivated GPS in the medium, as a practical measure to decrease the anticomplementary activity of tissue culture fluids was first proposed by von Zeipel(9). Preliminary experiments demonstrated that rubella virus attained similar titers in cultures maintained with media containing either horse serum or GPS but the harvested fluids with the former were highly anticomplementary while those containing GPS were practically devoid of that effect.

Unfortunately after the concentration necessary to bring the antigenic potency to levels adequate for complement fixation assays the materials exhibit a moderate degree of anticomplementary activity. Although this is within the limits that can be compensated for by a small excess of complement it is still a practical disadvantage of the antigens prepared so far.

The yield of material containing 2-3 antigen units per 0.025 ml drop has been of the order of 6-9 ml per bottle of 2.3×10^7 cells (1/6th to 1/9th of the volume of tissue culture fluid harvested). Supernatant fluids of infected cultures can be therefore a practical source of antigen for routine serological work. Furthermore the preparations obtained are clear homogeneous proteinaceous solutions which permit greater accuracy in measurement than a disrupted-cell antigen in the complement fixing test and also seem advantageous in studies toward characterization of this soluble antigen.

The microtechnique employed can be adapted for the use of microplates and diluting loops and it seems most likely that the same antigens could be employed in other microtechniques for complement fixation.

The titers observed in convalescent patients are in the range of 1:8 to 1:64 and therefore similar to those reported by Sever and collaborators(1) with "packed cells" antigen preparations. The rise of antibody titers in early convalescence is not rapid and occasional samples obtained by the end of the

second week after onset of rash were found with titers of 1:4. In the group of patients presented in Fig. 3 all the specimens collected during the fourth week after onset of rash had 2-fold or greater increase over the titers of the acute phase sera. Peak titers seem to obtain about the third month and persist without change, or with only slight decline throughout the first year following infection.

Sever *et al*(1) and Stern(2) have found that patients who do not recall any recent infection and who have negative complement fixation titers often have, however, good titers of neutralizing antibody. The complement fixation assay seems therefore to be most valuable in diagnosis of recent infection. One situation in which this characteristic could be used advantageously is the screening of newborns suspected of harboring the virus, since testing of the mother's serum could rapidly indicate those infants in whom virological studies should be conducted.

It was of interest to note that on filtration through Sephadex G-200 the fraction with the greatest antigenic activity followed the protein peak. Since previous experiments had demonstrated that infective virus appears in the first fractions which follow the void volume(10) this suggested that at least part of the antigenic activity was due to units smaller than the virus. This was confirmed by the fact that the antigenic activity of fractions previously purified by DEAE fractionation could be demonstrated after filtration through millipore filters of 50 and 10 mμ while it has been reported that infective virus is retained by filters of 100 and 50 mμ pore diameter (11).

Experiments are now in progress to quantitate the participation of this filterable component in the total antigenic activity and to test its reactivity by other immunological methods and its antigenicity in experimental animals.

Summary. Homogeneous preparations of rubella complement fixing antigen, in sufficient quantity for routine serological work, have been obtained from the fluid phase of infected tissue cultures. Filtration experiments have demonstrated that at least part of the antigenic activity is due to units smaller than the virus particles. Additional experiments on purification and characterization of this material are in progress. Complement fixing antibodies appear slowly after infection, reaching their peak titers by the third to sixth month, and persist with only a slight decrease a year after the acute episode.

ADDENDUM: N. J. Schmidt and E. H. Lennette (Proc. Soc. Exp. Biol. & Med., 1966, v121, 243) recently demonstrated a similar soluble antigen derived from tissue culture infected with rubella virus.

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