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Received October 5, 1966. P.S.E.B.M., 1967, v124.

## Rubella Virus Neutralization by Plaque Reduction.* (31692)

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Rubella virus assay in green monkey kidney cells utilizing the interference to enterovirus cytopathic effect (CPE)(1) has provided substantial information about the nature of this agent(2). The direct cytopathic effect of the virus on human amnion cells(3) and on cell lines derived from rabbit tissues has also been found useful in rubella virus assays (4,5,6). Interference with CPE of challenge enteroviruses(7,8,9) and Sindbis virus(10) by rubella virus has been used also to assay neutralizing antibodies to rubella virus. However, the test requires at least 7 days and the titers of antibodies obtained are dependent on a number of factors which include rubella virus dose, day of addition of challenge virus and presence of antibodies in the test sera not only to rubella virus but also to the challenge virus(7,10). Rubella virus antibody assay by the prevention of direct CPE also requires at least 7 days and requires the use of virus adapted to the cell line used(6,11,12).

The development of a hemadsorption-negative plaque assay(13) has provided a means of studying the neutralization of rubella virus more quantitatively. The present study was undertaken to develop a rapid method of detecting and quantitating rubella antibodies and to determine some of the parameters of neutralization.

*Materials and methods. Tissue culture and media.* African green monkey kidney (GMK) cells were cultivated by a technique previously described(14). BSC-1 cells(15) and $BHK_{21}$ cells(16) were grown in 16-oz prescription bottles. Cells were grown in Eagle's basal medium supplemented with 10% fetal bovine serum, antibiotics (penicillin 100 units/ml and streptomycin 100 $\mu$g/ml) and 1.5 g bicarbonate/liter. Cells grown in plastic petri dishes were placed in the medium described above but containing 2% fetal bovine serum, 2.25 g/liter of bicarbonate and in addition, 50 units/ml of mycostatin. Serum and virus dilutions were made in tris buffer (pH 7.2) supplemented with 2% fetal bovine serum for virus stabilization(2).

*Viruses.* The strains of rubella virus used were isolated from thyroid (R-1) and lung (R-3) tissues of infants with congenital

* Aided by USPHS grants AI-05382 and 5T1 AI-74 and USPHS Fellowship 1-F3-AI 25,943, from Nat. Inst. of Allergy and Infect. Dis., Nat. Inst. Health.
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rubella and identified with hyperimmune rabbit antiserum(17). Stock virus was prepared in monolayers of $BHK_{21}$ cells in 16-oz prescription bottles. Monolayers of cells were drained and inoculated with 1 ml of seed virus. After 1 hour absorption, medium was added and the cells were incubated at 37°C. The medium was changed twice weekly and the virus-containing fluids were harvested on the 14th day. Cell debris was removed by low speed centrifugation and the virus was stored at —90°C in sealed glass ampoules. Newcastle disease virus (NDV), originally from the American Type Culture Collection, was inoculated into the chorioallantoic sac of 9-10-day-old embryonated hen's eggs. The chorioallantoic fluid was harvested after 48 hours of incubation at 37°C and stored at —90°C in ampoules. NDV stocks titered $3 \times 10^8$ plaque forming units/ml on chick embryo fibroblast cells. Echovirus 11 (Gregory) was obtained from the World Health Organization Enterovirus Reference Centre, Houston, and passed in GMK cells.

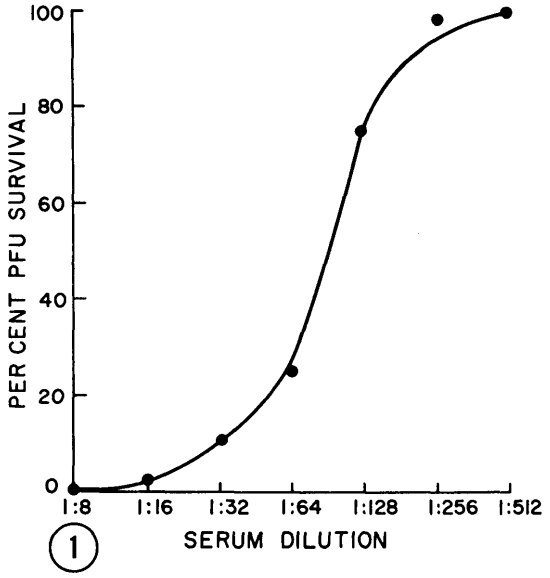
*Virus assay.* Rubella virus was assayed utilizing the interference in GMK cells with CPE of echovirus 11(2) and by a modification of the hemadsorption-negative plaque test described by Marcus and Carver(13). The hemadsorption-negative plaque test was performed in monolayers of BSC-1 cells grown in 60 mm plastic petri dishes (Falcon Plastics, Los Angeles, Calif.) Twenty-four-hour-old monolayers of BSC-1 cells were inoculated with 0.2 ml of the sample to be assayed. The virus was allowed to adsorb for 1 hour at 37°C in a 5% $CO_2$ atmosphere. The monolayers were flooded with 4 ml of medium and incubated for 48 hours, after which 0.2 ml of a 1:4 dilution of NDV virus stock was added. The monolayers were incubated an additional 16-20 hours at 37°C. The medium was removed and 2.0 ml of a 0.5% suspension of sheep red blood cells in saline was added. Within 20 minutes at room temperature a confluent sheet of red blood cells adsorbed to the monolayers except in areas of cells infected with rubella. These areas were readily counted by inspection. Echovirus 11 was assayed as previously described(18).

*Antisera.* Human sera were collected within 3 months after birth, from infants born with congenital rubella. The mothers of these infants were also bled soon after the birth of the infants. Sera were also obtained from randomly selected females of ages from 6-35 years. The sera were stored at —20°C until use. The preparation of rubella rabbit antiserum has been previously described(17).

*Complement.* Young adult guinea pigs were exsanguinated by cardiac puncture. The serum collected was pooled, sealed in glass ampoules and stored at —90°C. The complement was titered(19) using commercially prepared hemolysin (Baltimore Biological Laboratories, Baltimore, Md.). Repeated determinations revealed that the serum pool used contained 110 hemolytic units of complement/ml. Some lots of guinea pig serum were found to have a heat stable inhibitory effect on rubella virus when used at high concentrations.

*Neutralization test.* Based on previous titrations of the stock, rubella virus was diluted to contain approximately 100 plaque forming units (PFU) per 0.2 ml. Two-fold dilutions of the sera to be tested were made and equal volumes of the virus and serum dilutions were mixed. The mixtures were incubated at 37°C for 30 minutes or 1-2 hours at room temperature. The surviving virus was then assayed by the negative-hemadsorption plaque test. In some instances aliquots of the mixtures were also inoculated into 3 tubes of GMK cells which were subsequently challenged with echovirus 11 after 6 days of incubation at 37°C. In experiments in which guinea pig serum was added, the mixtures contained equal volumes of virus, test serum, and heat-inactivated (56°C for 30 minutes) or unheated guinea pig serum. In each test, the following controls were present: uninfected cells, serum without virus, virus without antiserum, and titration of the virus.

*Results.* Hyperimmune rubella antiserum prepared in a rabbit was used to study the effect of antibody concentration on rubella virus survival. The results of a typical experiment are shown in Fig. 1. Slight reduction of PFU occurred at a serum dilution of 1:128, but 4 times this amount of antibody yielded a



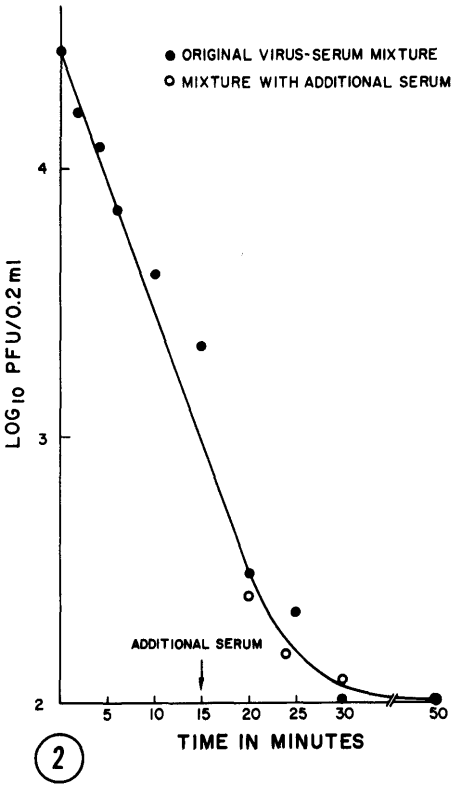




FIG. 1. Effect of rubella antibody concentration on virus survival.
FIG. 2. Kinetics of neutralization of rubella virus at 37°C.

90% reduction of virus, thus demonstrating a relatively narrow band of neutralization. Similar curves were obtained repeatedly with this and other sera. The serum dilution producing a 70% reduction in PFU was taken as the neutralization endpoint. A reference serum prepared by pooling 5 sera from adult convalescent patients was titered on 5 occasions. On 3 occasions a 70% reduction in PFU count occurred at a dilution of 1:32 and on 2 occasions the same endpoint was obtained at a dilution of 1:64. These results suggest that rubella antibody determination by the plaque reduction method gives a sharp and reproducible endpoint.

The time necessary for the virus-antibody reaction to occur was examined at 37°C. Undiluted rubella virus was mixed with an equal volume of undiluted human reference serum and incubated in a 37°C water bath. At intervals aliquots were removed, immediately diluted in cold diluent and assayed for surviving virus. The results of one such experiment are presented in Fig. 2. The virus-antibody reaction was essentially complete after 30 minutes of incubation. The addition of more antibody after 15 minutes of incubation did not alter the virus survival, indicating that the original virus-antibody mixture contained an antibody excess. Similar results were obtained in 2 repeat experiments. Plots of the logarithm of virus survival against time resulted in straight lines passing through the origin thus indicating first order kinetics of the antibody-virus reaction similar to that found with other viruses(20).

A number of viruses have been shown to be neutralized more efficiently by addition of fresh serum components(21,22,23). Recent studies with herpes simplex virus have shown that antibodies produced early in the course of immunization are complement dependent (24). Experiments were undertaken to determine the effect of guinea pig complement on rubella virus neutralization. As reported by Leerhoy(5), it was found that heating sera at

TABLE I. Effect of Complement on Rubella Antibody Titer.

| Serum No. | Antibody titer: Heated GP serum | Unheated GP serum | Fold enhancement |
|---|---|---|---|
| 1* | 1:32 | 1:256 | 8× |
| 1* | 1:32 | 1:256 | 8× |
| 2 | 1:16 | 1:256 | 16× |
| 3 | 1:32 | ≥1:512 | ≥16× |
| 4 | 1:16 | 1:64 | 4× |
| 5 | 1:16 | 1:256 | 16× |
| 6 | 1:16 | 1:256 | 16× |
| 7 | 1:32 | 1:128 | 4× |
| 8 | 1:32 | 1:128 | 4× |

* Separate determinations.

Sera 1-3: from infant with congenital rubella under 1 mo of age. Sera 4-6: from mother of infant with congenital rubella; obtained about 1 yr after rubella infection. Sera 7 and 8: from adult with history of rubella more than 5 yr before blood obtained.

56°C for 30 minutes reduced the rubella antibody titers. The addition of unheated guinea pig serum increased the titers by varying degrees. Table I summarizes the antibody titers obtained upon addition of guinea pig serum containing 10 hemolytic units of complement to heat inactivated human sera-virus mixture. It can be seen that a 4-fold to 16-fold enhancement was observed in all sera tested. Enhancement of the antibody titers occurred in sera obtained both from individuals with recent rubella infection and from individuals with remote histories of rubella.

The amount of guinea pig serum necessary to enhance rubella antibody titers was determined by observing the effect of decreasing concentrations of heat-inactivated and unheated guinea pig serum. The heat-inactivated guinea pig serum did not enhance the rubella antibody titers at the highest concentration tested. However, as can be seen in Table II, increasing amounts of unheated guinea pig serum produced increasing amounts of enhancement. Serum containing as little as 2.5 hemolytic units of complement had an enhancing effect.

The heat lability of the enhancing effect suggested that the active principle might be complement. An experiment was designed to remove complement by a heterologous antigen-antibody reaction. This was performed by adding 100 μg crystalline egg albumin in 0.2 ml (Sigma Chemical Co, St. Louis, Mo.) and rabbit anti-egg albumin in equivalent proportions (as determined by the quantitative precipitation reaction) in 0.2 ml to 0.2 ml of fresh unheated guinea pig serum. The mixture was allowed to react overnight at 4°C after which the precipitate was removed by centrifugation at 1500 rpm for 30 minutes in a refrigerated centrifuge. The supernatant fluid was titered for complement activity and also tested for the ability to enhance rubella antibody titers. Table III summarizes the results of duplicate experiments and it can be seen that the removal of complement by the heterologous antigen-antibody system also removed the rubella antibody-enhancing effect.

The plaque reduction method was compared with the tube neutralization test, which is dependent upon interference with echovirus 11, by simultaneously assaying randomly selected human sera at 2 serum dilutions. The results are presented in Table IV. Eleven per cent (6 of 54) of the sera which were negative for antibody at a serum dilution of 1:8 by the tube neutralization method were found to contain antibody by the plaque re-

TABLE II. Effect of Complement Concentration on Rubella Antibody Titers.

| Hemolytic units of complement | Exp 1 — Titer: With heated GP serum | Exp 1 — Titer: With unheated GP serum | Exp 1 — Fold enhancement | Exp 2 — Titer: With heated GP serum | Exp 2 — Titer: With unheated GP serum | Exp 2 — Fold enhancement |
|---|---|---|---|---|---|---|
| 20 | 1:32 | 1:256 | 8× | 1:16 | 1:256 | 16× |
| 10 | NT* | 1:128 | 4× | 1:16 | 1:128 | 8× |
| 5 | NT | 1:128 | 4× | 1:16 | 1:128 | 8× |
| 2.5 | NT | 1:64 | 2× | 1:16 | 1:64 | 4× |
| 1.25 | NT | 1:32 | 0 | 1:16 | 1:32 | 2× |
| 0 | — | 1:32 | — | — | 1:16 | — |

* Not tested.

TABLE III. Effect of Heterologous Antigen-Antibody System on Enhancing Effect of Guinea Pig Serum.

| Components of reaction* | Results: Hemolytic units of complement Exp 1 | Exp 2 | Antibody titers Exp 1 | Exp 2 |
|---|---|---|---|---|
| Anti-EA, EA, C′ | 0 | 0 | 1:16 | 1:16 |
| Anti-EA, EA, Tris | 0 | 0 | 1:16 | 1:16 |
| Control (Tris & FBS) | 0 | 0 | 1:16 | 1:32 |
| EA, C′, Tris | 10 | 10 | 1:128 | 1:128 |
| Anti-EA, C′, Tris | 5 | 8 | 1:128 | 1:128 |
| C′, Tris | 10 | 10 | 1:64 | 1:128 |

* Equal parts of rabbit anti-egg albumin (Anti-EA), egg albumin (EA) and guinea pig serum complement (C′) reacted overnight at 4°C. The precipitate was removed and the clarified guinea pig serum titered for rubella antibody enhancing effect and hemolytic activity.

duction method. Twenty sera were also examined at a dilution of 1:32 and 3 of these sera were negative for antibody by the tube neutralization test but reduced the plaque count by 70%. The results of this comparative study suggest that the plaque reduction method may be a more sensitive means of detecting rubella antibodies.

*Discussion.* Methods for measurement of rubella neutralizing antibodies have been described which depend upon the prevention of interference with enterovirus CPE in GMK cells(7,8) or upon the prevention of the direct CPE of rubella in primary human amnion cells(3,10) in $RK_{13}$ cells(11,12) in LLC-$RK_1$ cells(6), or in SIRC cells(5). The antibody titers obtained utilizing the interference with echovirus 11 CPE are dependent on such factors as the day of challenge with echovirus 11, the dose of rubella virus used, the addition of fresh rabbit serum, the presence of echovirus 11 antibodies in the test sera, and the use of partial or complete interference as the endpoint(7,10). The use of $RK_{13}$ or other rabbit cell lines requires the use of a virus strain adapted to these cells (6,12) and gives lower antibody titers than obtained in SIRC cells(5). All methods thus far described require 7 or more days to complete the antibody assay.

The hemadsorption-negative plaque assay for rubella virus in BSC-1 cells provides quantitation and is easily performed. The neutralization test described here utilizing the hemadsorption-negative plaque assay has the advantage of being both quantitative and rapid, requiring only 3 days. The virus dose employed is less critical. The variabilities introduced by day of challenge and interpreting the amount of interference present as an endpoint are overcome. It is possible that the technique may also be applied to study antigenic variations of virulent and candidate vaccine strains of virus by demonstrating differences in the kinetics of neutralization.

A number of viruses have been shown to be neutralized more effectively by addition of fresh serum components(21,22,23). Herpes simplex virus is more efficiently neutralized by antibodies produced early in the course of immunization if complement is added(24). Rubella virus antibody titers are enhanced with fresh guinea pig serum and the factor or factors responsible for such enhancement are heat labile and are co-precipitated by a heterologous antigen-antibody reaction. These results suggest that complement is also instrumental in enhancing rubella virus antibody titers. Rubella antibody enhancement by fresh rabbit serum(3,7,10) may also be due to complement. The effects of comple-

TABLE IV. Comparison of Rubella Antibody Titers Obtained by Plaque Reduction and Tube Neutralization Test.

| Results | Serum dilution 1:8 Plaque reduction | 1:8 Tube neutralization | 1:32 Plaque reduction | 1:32 Tube neutralization |
|---|---|---|---|---|
| No. with rubella antibody | 35 | 29 | 4 | 1 |
| No. without rubella antibody | 19 | 25 | 17 | 20 |
| No. and % with antibody by plaque reduction but not by tube neutralization* | 6 (11%) | | 3 (14%) | |

* In no instance was antibody detected by tube neutralization which was not found by plaque reduction.

ment on rubella virus antibodies and herpes simplex antibodies appear to differ, in that antibodies produced long after immunization are not enhanced in the herpes simplex system but are enhanced by complement in the rubella virus system. Further studies will be necessary to determine the action of complement in the enhancement of rubella virus antibody titer.

*Summary.* The hemadsorption-negative plaque assay technique has provided a means of quantitatively studying rubella virus neutralization. A 3-day assay for rubella antibodies has been developed which is sensitive and reproducible. The addition of fresh guinea pig serum enhanced rubella antibody titers up to 16 times those obtained in heated sera. The factor or factors responsible for the enhancement of antibody titers have the properties of complement.

The authors gratefully acknowledge the able assistance provided by Miss Marian Moore and Mrs. Marjorie Burkhardt. The helpful advice and consultation of Dr. Gordon R. Dreesman is deeply appreciated.

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Received October 6, 1966. P.S.E.B.M., 1967, v124.

## A Comparison of Alkylhydrazines and Their $B_6$-Hydrazones as Convulsant Agents.* (31693)

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The rocket propellants 1,1-dimethylhydrazine (UDMH) and monomethylhydrazine (MMH) are powerful convulsant agents(1, 2,3,4). Data are available on the acute

* This study was supported by U. S. Air Force Contract AF 33(615)-2332. Further reproduction of this material is authorized to satisfy the needs of the United States Government.