in all positive sera. This antibody reacted with *M. pneumoniae* ID antigen to form a complex which accepted a lipid as well as a protein stain. Additional bands formed in reactions with some sera and antigen accepted only protein stain. Other investigators have speculated that the growth inhibiting antibody to *M. pneumoniae* is directed against an antigen that contains lipid (19). Since there is good correlation between antibodies detected in ID and TRI tests, the present findings support the assumption that a lipid component is involved in the formation of growth inhibiting antibody.

In addition to its specificity and sensitivity, the ID test is a rapid, easy method for the detection of human antibody to *M. pneumoniae*.

*Summary.* *M. pneumoniae* antibodies in human sera are detectable by double diffusion gel precipitation. These antibodies correlate with growth inhibiting antibody measured by the tetrazolium reduction inhibition test. All positive sera share a common precipitation band which appears to contain lipid.

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## Fluorescent Plaque Assay of Rubella Virus Infectivity (33332)

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The need for a rapid and precise plaque assay for quantitating rubella virus infectivity has long been recognized. Several plaque tests have been described earlier (1–9), but thus far no single infectivity assay has been applied with uniform ease and advantage from one laboratory to another. Most of these procedures require a relatively long period of cell viability under agar and the use of virus adapted to the assay cell system by serial passage.

In this report a fluorescent focus assay

system is described. This method, which has been found useful in measuring the infectivity titers of several other viruses (10–16), is based on the indirect Coons fluorescent antibody technique. The assay provides reproducible infectivity values within 72 hr.

*Materials and Methods. Viruses.* Rubella strain M-33 (17) was used both in third BS-C-1 (18) passage and in seventy-seventh primary African green monkey kidney cell passage. Strain Putnam (19) had been passaged 12 times in continuous $RK_{13}$ rabbit kidney cells (20), once in MA-104 (Microbiological Associates) and 4 times in BHK-21 (21) cell cultures.

*Cell cultures.* Continuous $RK_{13}$ rabbit kidney cells were seeded into plastic petri dishes (35 × 15 mm, Falcon Plastics Company, Los Angeles, California) at $2 \times 10^5$ cells/dish, and the dishes were incubated at 36° in a humidified atmosphere of 5% $CO_2$ in air. The cultures were inoculated with virus 24 hr later when 40–60% of the petri dish area was covered by cells. Growth medim was Eagle's MEM containing 5% fetal bovine serum, penicillin, and streptomycin. Primary African green monkey kidney cell cultures (GMK) were used for the interference assay of rubella virus infectivity.

*Inoculation of cultures.* Growth medium was removed by aspiration, and ordinarily 0.05 ml of virus was added to each culture with a 0.10 ml serologic pipette. The dishes were placed on a shaker specially designed to spread the small volume across the relatively large dish every 30 sec (22). The work reported in this paper was done with the aid of a mechanical shaker, but satisfactory results can be obtained by shaking the cultures manually (16). After being shaken at room temperature for 10 min, the cultures were fed with 3.0 ml of growth medium and reincubated for 72 hr.

*Fixation.* The medium was removed by aspiration and each dish was washed 4 times with 2.0 ml of 0.85% saline. The cells were fixed for 4 min with 95% methyl alcohol and washed 2 times with 2.0 ml of 0.01 *M* phosphate buffered saline, pH 7.2. Cell monolayers were either stained immediately or stored at −20° or −70° for later staining.

*Antisera.* Acute and convalescent sera were obtained from a patient with clinical rubella. Early convalescent sera obtained from Fort Ord, U. S. Army recruits, was supplied through the courtesy of Dr. Earl S. Beck of the National Institute of Allergy and Infectious Diseases, NIH. The rubella hemagglutination–inhibition titers of the convalescent sera ranged from 1:256 to 1:1024.

Hyperimmune guinea pig serum was produced by inoculating purified Putnam strain rubella antigen into the foot pads of guinea pigs at weekly intervals for 5 weeks. The antigen was grown in BHK-21 cell cultures and was banded in a linear cesium chloride density gradient as described previously (23). The band of virus was removed through a needle hole in the side of the gradient tube and dialyzed against phosphate buffer to remove the cesium chloride. Each 0.1-ml dose of antigen contained roughly $10^5$ fluorescent plaque units (fpu) of rubella virus. The antisera produced in this way had rubella hemagglutination–inhibition titers of 1:256 and 1:512.

Horse antihuman gamma globulin conjugated with fluorescin isothiocyanate (FITC) was obtained from Roboz Surgical Instrument Company, Washington, D. C.

Antiguinea pig globulin of rabbit origin conjugated with FITC was obtained from Colorado Serum Company, Denver, Colorado.

*Staining.* 0.1 ml of rubella antiserum was added to each dish. The dishes were incubated for 30 min at room temperature on the mechanical shaker. The antiserum was removed, and the monolayers were washed 6 times with 0.01 *M* phosphate buffer, pH 7.2. One-tenth ml of FITC conjugated antiglobulin diluted 1:5 in pH 7.2 phosphate buffer was added, and again the dishes were shaken for 30 min. The cells were then washed 3 times with the phosphate buffer and examined for fluorescence.

*Examination of cultures.* A Bausch and Lomb microscope fitted with a paraboloid dark field condenser was used. The source of UV light was an HBO 200 high pressure mercury arc lamp. Barrier filter T2 and exciter filters 5-58 or 7-51 were used. Counting

was done with the aid of an eyepiece grid so that a known area of the cell monolayer could be viewed for easy quantitation (16). At 35 × this area was 2.2 mm². The mean number of fluorescent plaques (FP) counted per grid area was calculated after counting at least 30 FP to reduce sampling error. This was then multiplied by a constant (area of the petri dish/area of the gridded space) to determine the number of FP on the dish. This number was taken as the number of rubella virus infectious units in the inoculum. Calculations were made to express the titers per milliliter by multiplying by 1.0/volume of inoculum and by 1.0/virus dilution, as follows:

$$\text{FP/1.0 ml} = \frac{\text{no. of FP counted}}{\text{no. of grid areas counted}} \times \frac{\text{area of petri dish}}{\text{area of gridded space}} \times \frac{1.0}{\text{vol of inoc.}} \times \frac{1.0}{\text{virus dilution}}.$$

*Results.* In the first week after inoculation of rubella virus onto $RK_{13}$ cell monolayers, infected cells were morphologically indistinguishable from uninfected cells. Approximately 40 hr after inoculation, however, plaques consisting of 10–30 cells showing granular, cytoplasmic fluorescence appeared in infected $RK_{13}$ cell cultures. Plaque size was quite uniform within each experiment in the 30–72 hr period after virus inoculation, but did vary from experiment to experiment. Cultures were routinely fixed and stained approximately 72 hr after infection. There was no suggestion that secondary plaques had formed at this time, although rarely a single fluorescing cell could be seen between plaques.

*Specificity of staining.* Uninfected $RK_{13}$ cell controls were included in every experiment and never showed specific fluorescence. When rubella infected cell cultures were stained with acute serum from a patient with rubella, there was no specific fluorescence. In contrast, convalescent serum from the same patient regularly stained infected cells at dilutions of up to 1:20. In addition, when infected $RK_{13}$ cell cultures were stained with only antihuman or antiguinea pig gamma globulin conjugated with FITC, only tissue autofluorescence was seen. When convalescent serum was added to cultures after the 10-min virus adsorption period, the plaque count was reduced 97%.

TABLE I. FP/ml Obtained Using Various Inoculum Sizes.[a]

| | Inoculum size (ml) | | | | |
|---|---|---|---|---|---|
| Expt. no. | 1.0 | 0.5 | 0.1 | 0.05 | 0.025 |
| 1 | 3.48 (2) | 3.79 (2) | 4.21 (2) | | |
| 2 | | | 4.95[b] (10) | 5.25 (10) | |
| 3 | | | | 3.39[c] (8) | 3.33 (10) |

[a] Titers shown represent the means of each group of replicate cultures; the number of cultures in each group is given in parentheses.

[b] In this experiment the mean titers are significantly different ($p < .05$) while there is no significant difference in the group variances ($p > .05$).

[c] There was no significant difference ($p > .05$) in either the mean titers or group variances in this experiment.

*Optimum volume for inoculation.* Experiments were designed to determine the ideal volume of inoculum for plaque assay. In different experiments various volumes of virus ranging from 1.0 to 0.025 ml were inoculated onto $RK_{13}$ cell monolayers, and shaken for 1 hr at room temperature. The inocula were then removed and maintenance medium added. The cultures were fixed and stained as described above. Of the range of inoculum volumes tested, the smallest, 0.025 and 0.05 ml, yielded the highest titers; larger inocula up to 1.0 ml yielded lesser titers (see Table I). There was no significant difference between the titers obtained with 0.025 and 0.05 ml inocula. Significantly higher titers were obtained with inocula of 0.05 ml as opposed to 0.10 ml inocula, but the variances in titers within these two groups were small and not significantly different. The inoculum volume of 0.05 ml gave maximum titers and greatest precision and was therefore chosen as the most efficient volume for routine use.

*Dose–response relationship in fluorescent*

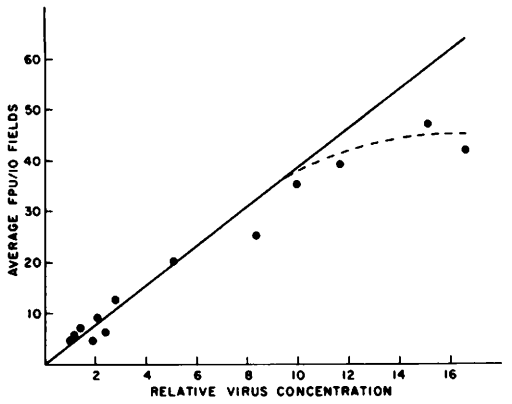


FIG. 1. The dose–response line of fluorescent plaque assay is straight except where plaques are so numerous they tend to coalesce making plaque counts disproportionately low (— — —).

*plaque assay.* The linear relationship between the concentration of the rubella virus in the inoculum and the fluorescent plaque count is shown in Fig. 1. Thirteen dilutions of virus seed were prepared, and each was inoculated onto an $RK_{13}$ cell monolayer. After a 10-min adsorption period at 22°, the cultures were washed, fed with 3.0 ml of growth medium, and incubated for 72 hr at 36°. Plaques were counted and plotted against relative virus concentration. The linear relationship between virus concentration and fluorescent plaque count held over a range of 1.0 $\log_{10}$ units. When the titer of the original undiluted virus seed was calculated from the FP counts made at the various dilutions, the standard deviation of the entire group of 13 titers was 23% of the mean. The most precise counts were those in the midconcentration range, as might be expected. At the highest concentration, plaques tended to merge, and counting was more difficult; consequently the values were disproportionately low.

*Sensitivity and precision.* Comparisons made between titers obtained by the fluorescent plaque technique and the conventional interference assay in tube cultures of GMK cells have shown close agreement in titers between systems. Table II shows the results of repeated titrations on aliquots of 2 virus pools. The M33 strain rubella virus shown in the table represents low passage virus which is not adapted to $RK_{13}$ cells; the $TCID_{50}$ and FP titers of this strain were equivalent. Also in agreement were the titers of Putnam strain virus which had been passed 12 times in $RK_{13}$.

TABLE III. Precision of Fluorescent Plaque Assay for Rubella Virus.

| Culture no. | Total fluorescent plaques/total fields counted | Calculated no. of fluorescent plaques/ml of inoculum ($\times 10^5$) | $Log_{10}$[a] |
|---|---|---|---|
| 1 | 126/30 | 1.51 | 5.18 |
| 2 | 139/31 | 1.61 | 5.21 |
| 3 | 135/30 | 1.62 | 5.21 |
| 4 | 180/32 | 2.03 | 5.31 |
| 5 | 171/30 | 2.05 | 5.31 |
| 6 | 127/30 | 1.52 | 5.18 |
| 7 | 189/31 | 2.19 | 5.34 |
| 8 | 150/31 | 1.74 | 5.24 |
| 9 | 157/31 | 1.82 | 5.26 |
| 10 | 146/34 | 1.55 | 5.19 |

[a] Mean 5.25, SD .06.

In one of a series of experiments designed to determine the precision of the fluorescent plaque assay, 10 titrations were done simultaneously on the same pool of virus (see Table III). The standard deviation of these titers, 0.06 $\log_{10}$ FP/ml, indicates that the precision of this technique is excellent. In ad-

TABLE II. Sensitivity of Fluorescent Plaque Assay for Rubella Virus.

| Virus | Titration method | Titration results[a] | Mean | SD |
|---|---|---|---|---|
| M33 BS-C-$1_3$ | $TCID_{50}$ | 5.1, 5.1, 5.1, 4.5, 4.5, 4.3 | 4.77 | .37 |
| | FP | 5.0, 5.0, 5.0, 5.0, 4.9, 4.8, 4.8, 4.5 | 4.88 | .17 |
| Putnam | $TCID_{50}$ | 4.1, 3.9, 3.5, 3.5, 3.5, 3.4 | 3.65 | .28 |
| $CRK_{12}$ $MA104_1$ $BHK_1$ | FP | 3.9, 3.8, 3.7, 3.6, 3.6 | 3.72 | .13 |

[a] $Log_{10}$ $TCID_{50}$/ml or $\log_{10}$ FP/ml.

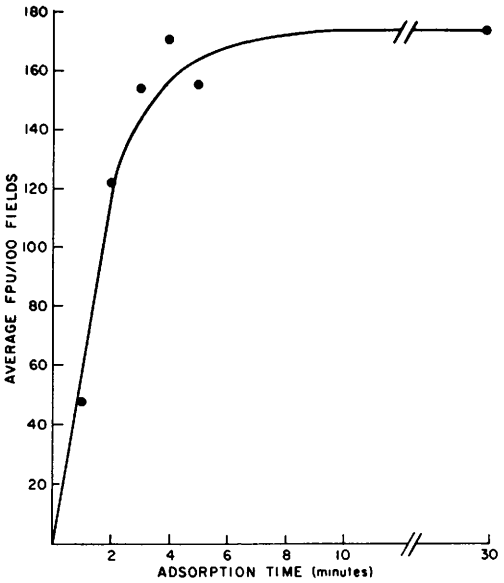


FIG. 2. The number of fluorescent plaques increased rapidly in the first 4 min of the virus adsorption period but was stable thereafter.

dition, the smaller standard deviations of the FP titers in Table II are a reflection of the greater precision of the plaque assay.

*Optimum time for adsorption.* The rate of rubella virus adsorption to $RK_{13}$ cell monolayers was studied. Cultures were inoculated with 0.05 ml aliquots of a virus pool, and the inocula were allowed to adsorb for varying periods of time ranging from 1 to 30 min. The inoculum was removed by aspiration, medium was added, and the cultures were incubated for 72 hr. The number of fluorescent plaques increased rapidly during the first 4 min, but afterward the titer remained relatively constant (Fig. 2).

*Discussion.* The fluorescent plaque assay depends on the production of foci of cells containing rubella antigen. Since this phenomenon is demonstrable within 3 days of inoculation, results are available much more rapidly than in titrating systems dependent on cytopathic effect or interference. No agar overlay or culture medium change is necessary within this short period, eliminating risks of contamination.

It is not surprising that smaller volumes of inocula were more efficient in this technique (24, 25). Volumes of 0.05 ml yielded the highest titers while allowing excellent precision in the assay. It was possible to deliver 0.05 ml from a 0.10 ml serological pipette with acceptable accuracy and to distribute this inoculum evenly over the entire cell monolayer. The lower titers obtained from larger volume inocula probably resulted from a small percentage of the infectious particles making contact with the host cells in the allotted adsorption period and, probably less important, loss of unadsorbed virus through thermal inactivation during this period.

The dose–response line is straight throughout most of the range shown in Fig. 1 demonstrating that a single virus unit is sufficient for infection in this system (26). The best precision is achieved when cultures with roughly 1–2 plaques/field are counted. Precision is good over a wide range of average plaque per field values so that cultures containing from 200–1200 plaques are suitable for quantitation.

Adsorption of over 90% of the inoculated rubella virus was achieved in 4 min. This adsorption is surprisingly rapid even considering that the virus has a relatively small diameter of 55 mμ (23) and that the inoculum was small.

It is always desirable to identify an interfering or cytopathic agent as rubella virus by performing a typing neutralization test. This identification is simplified by the inherent specificity of the FP system where acute serum does not stain infected cells but convalescent serum does.

Fluorescent plaques were produced by rubella virus of both high and low tissue culture passage history. Virus did not have to be adapted to $RK_{13}$ cell culture in order to be titrated by the fluorescent plaque assay.

*Summary.* Plaques of rubella virus infected cells demonstrating granular, cytopathic fluorescence were counted in $RK_{13}$ cell cultures fixed 72 hr after infection. There was a linear relationship between virus concentration and fluorescent plaque count. Counts on replicate cultures have demonstrated that the technique is very precise. The sensitivity of the fluorescent plaque assay was equal to that of the conventional GMK-interference titration method.

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# Experimental Thyroiditis in Complement Intact and Deficient Mice Following Injections of Heterologous Thyroglobulins without Adjuvant* (33333)

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Experimental thyroiditis has been induced in rabbits by injections of aqueous preparations of altered homologous (1) and certain heterologous thyroglobulins (2, 3). In either situation, the production of thyroiditis appears to be the result of a termination of a natural unresponsive state to autologous thyroglobulin (4, 5). Aqueous preparations of thyroglobulin are rapidly catabolized and do not persist as a sustained stimulus as in the case of injections of thyroglobulin incorporated in complete Freund's adjuvant; thus a secondary response to a subsequent injection

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