Plaquing of *Toxoplasma gondii* in Secondary Cultures of Chick Embryo Fibroblasts

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Received for publication 14 October 1968

A tissue culture system that employed chick embryo fibroblasts was described for plaquing of the obligate intracellular parasite *Toxoplasma gondii*. High plaquing efficiency and reproducibility were accomplished by the use of secondary rather than primary cultures and the use of toxoplasma obtained from disrupted peritoneal cells of mice infected 48 hr earlier. The monolayers were cultured in a special medium which maintained the fibroblasts until the maximal number of plaques was produced. Optimal plaque formation was obtained in 5 days, and the plaques were easily counted macroscopically.

In 1959, the plaque method originally described for the study of animal viruses by Dulbecco in 1952 (3) was adapted to the obligate intracellular parasite *Toxoplasma gondii* by Ch Paras and Schlesinger (2). To study quantitatively the effects of various agents on toxoplasma and host cell-toxoplasma relationships (5), it was found necessary to develop a plaquing method which produces reproducible results when submitted to the same environmental conditions and toxoplasma challenge and which produces large plaque size for accurate counting and identification. Such a method is described for the plaquing of toxoplasma in tissue culture.

**MATERIALS AND METHODS**

Fertilized eggs free from the avian leukemia virus group were obtained from Kimber Farms, Fremont, Calif. Primary cultures of chick embryo cells were prepared, by the method described by Dulbecco (3), from a pool of 9-day-old, decapitated embryos. The growth medium employed was a modification of a medium described by Rubin (6), consisting of two parts Tryptose Phosphate Broth (Difco), 1 part fetal calf serum, one part chick serum, one part sodium bicarbonate solution from a stock solution of 2.8%, 95 parts medium 199, and antibiotics to give a final concentration of 50 units of penicillin per ml and 100 μg of streptomycin sulfate per ml. Sera were inactivated at 56 C for 30 min prior to use, and only sera which were negative in the Sabin-Feldman dye test were used. A sample of 6 × 10⁶ cells was seeded in 100- by 20-mm petri dishes. The primary cultures were incubated at 37 C in a humidified atmosphere of 5% CO₂. After 48 hr a confluent cell layer formed, which was composed of a mixture of cell types, including macrophages. The primary cells were then either subcultured for use in the plaque assay or were maintained for future use by overlaying with soft agar medium. This latter medium was prepared from the growth medium described above by decreasing the content of medium 199 (1X) to 55 parts and by the addition of 20 parts of medium 199 (2X) and 20 parts of 1.8% Special Agar (Noble; Difco). The agar was pipetted in a volume of 10 ml per plate. The cell layers could be maintained under the soft agar for 7 days or subcultured during this period. For the purpose of subculturing, the agar was removed by tilting and gently shaking the dish. The cell layer was washed once with Earle's balanced salt solution (BSS) containing the antibiotics described above to remove all agar particles. The cells were then treated with 0.25% trypsin solution in phosphate-buffered saline (pH 7.4) for 10 min at 37 C. The detached cells were centrifuged for 2 min at 180 × g, the supernatant fluid was decanted, and the cells were resuspended in BSS. After being washed twice in BSS, the cells were seeded in 60- by 15-mm plastic petri dishes, each of which contained 5 × 10⁶ cells. Medium for secondary cultures, a modification of that described for the primary cultures, was prepared by increasing the Tryptose Phosphate Broth to 10 parts, the fetal calf serum to 4 parts, and by reducing medium 199 to 84 parts. After 48 hr a confluent monolayer composed of uniform fibroblast cells was formed which contained approximately 2.7 × 10⁶ cells per dish.

The RH strain of toxoplasma was obtained from the peritoneal cavities of 22- to 24-g female mice of the Swiss Webster strain which had been inoculated with parasites 48 or 72 hr earlier. A volume of 0.5 ml of collecting fluid (BSS with 20% fetal calf serum and 50 units/ml of preservative-free heparin) was inoculated into the peritoneal cavity. The exudate was then aspirated and collected in a tube containing additional collecting fluid. The tubes were kept in an ice bath at all times. To disrupt cells containing organisms, the preparation was forced through a
27-gauge needle by a syringe. The concentration of parasites was determined by counting dilutions of the suspension in a Neubauer-Levy hemacytometer. A sample containing the desired numbers of organisms was diluted in a suspension medium composed of 14 parts fetal calf serum, 14 parts chick embryo extract, 36 parts medium 199, and 36 parts BSS. The growth medium was removed from the cell layer and 2 ml of the suspension containing the desired number of organisms was pipetted onto the cell layer. After 1 hr of adsorption at 37 C, the medium was removed, and the monolayer was washed once with 5 ml of BSS containing 20% fetal calf serum. Agar overlay medium (composed of the same ingredients as was the soft agar medium but with 20 parts of a 4.75% solution of agar) was applied to each of the infected cultures in 5-ml portions. After the agar had solidified at room temperature, the cultures were incubated at 37 C in the 5% CO2 atmosphere for various time intervals. The cell layers were then stained with 3-ml portions per dish of 0.05% neutral red prepared in 0.15 m NaCl. After 2 hr incubation at 37 C, the stain was poured off and the plaques were counted macroscopically.

RESULTS

To determine the optimal time of harvest of toxoplasma for maximal plaquing efficiency, peritoneal fluids of mice infected either 48 or 72 hr earlier were used. The mice used 48 hr after they had been inoculated intraperitoneally with 6 x 10^6 organisms and those used 72 hr after inoculation with 4 x 10^6 organisms had been infected with organisms obtained from the peritoneal fluids of 3-day infected mice. Whereas toxoplasma obtained from mice infected 48 hr earlier gave a high plaquing efficiency and reproducible results, those obtained from mice infected for 72 hr gave a low plaquing efficiency and results which varied widely in repeated experiments. For this reason, only peritoneal fluids from mice infected for 48 hr were used in further studies. Optimal results in respect to

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>No. of parasites per dish</th>
<th>No. of plaques per dish</th>
<th>Avg plaque-forming units</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH-13</td>
<td>50</td>
<td>45, 51, 53</td>
<td>%</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>68, 71, 81, 85</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>Overlapping</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>Confuent plaquing</td>
<td></td>
</tr>
<tr>
<td>CH-14</td>
<td>50</td>
<td>31, 32, 32, 39, 42, 46,</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td></td>
<td>49, 50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>55, 57, 62, 69, 71, 71</td>
<td>64</td>
</tr>
</tbody>
</table>

* Read on the 5th day of incubation after infection.

FIG. 1. Toxoplasma plaques in a monolayer of chick-embryo fibroblasts. Organisms (100) from the peritoneal fluid of mice infected 48 hr earlier were used. The monolayer was stained on the 5th day after infection.

FIG. 2. Enlargement of toxoplasma plaques in monolayer of chick-embryo fibroblasts stained on the 5th day after infection. Note variation in size and morphology of plaques.
counting and reproducibility were obtained when 50 to 100 organisms were used. Larger numbers often resulted in overlapping of plaques. For instance, an inoculum of 600 parasites per dish resulted in almost confluent plaquing (Table 1). The plaquing efficiency in the two experiments shown ranged from 64 to 106%, the latter figure undoubtedly reflecting the error encountered in determining the inoculum size in the hemacytometer. Comparable studies of toxoplasma from mice infected for 72 hr revealed inconsistent plaquing efficiency of less than 25%.

Figure 1 shows a monolayer with plaques as they appeared 5 days after infection, and Fig. 2 depicts an enlargement of a group of plaques to show configuration 5 days after infection. Representation of the plaque areas by phase microscopy revealed cellular debris and free toxoplasma. The plaques were easily visible with the naked eye as white, irregular areas against a pink background of viable cells. When cultures were stained 4, 5, and 6 days after infection, the maximal number of plaques appeared consistently on day 5 and, thereafter, the plaques increased only in size. Intrapertioneal inoculation of material aspirated from the central areas of the plaques into mice resulted in the death of all animals due to systemic toxoplasmosis.

**DISCUSSION**

The use of secondary cultures of chick-embryo fibroblasts provides a uniform monolayer which gives clearly defined plaques of macroscopic size. In addition, the use of a soft agar medium to maintain the primary cultures allows for a supply of cells which can be maintained for 1 week or subcultured during this interval. The use of a modification of Rubin's agar overlay method for secondary cultures maintains the cells for a period of time necessary for plaques of optimal number and size to develop. In our study, this time period was 5 days. In contrast, the use of the primary cultures with the medium described by Chaparas and Schlesinger (2) often resulted in our laboratory in partial death of the monolayer, by the 5th day, and in plaques which were minute and had not reached maximal number by the 4th day.

Maximal plaquing efficiency and reproducibility were obtained by employing toxoplasma which had been released from lysed peritoneal cells. Extracellular parasites, of which the 72-hr fluid is mainly composed, cannot be relied upon to be infectious because it is not possible to determine the length of time they have been outside the host cell. Lycke and Lund (4) noted that if toxoplasma released from host cells do not immediately re-enter another cell, they begin to disintegrate. Chaparas and Schlesinger also noted greater efficiency when they used organsims obtained by artificial lysis of infected chick-embryo fibroblasts (2). The use of a reproducible plaque technique with at least 60% plaquing efficiency should allow for evaluation of various parameters of intracellular parasitism, with toxoplasma as the model organism. Isolation of individual plaques provides single clones of parasites, which can be used in studies of the molecular biology of the parasite as well as in studies of the differences in virulence, antigenicity, etc., within a single strain. The feasibility of such studies is suggested by the work of Akinshina (1).

**ACKNOWLEDGMENTS**

This investigation was supported by Public Health Service grant AI 04717 from the National Institute of Allergy and Infectious Diseases.

**LITERATURE CITED**