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Studies on *Toxoplasma gondii* in Tissue Culture
1. Susceptibility and Plaquing in a Human Leukemic Leucocyte Cell Line Culture (J-111)

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Introduction

Although multiplication of *Toxoplasma gondii* in tissue cultured cells has been reported by several groups of investigators, there are still many unsettled problems in the *in vitro* system. One of the problems is to assay the living organisms, quantitatively and reproducibly in an easy way. It has already been reported that the RH strain of *Toxoplasma gondii* induces the formation of necrotic plaques on monolayers of chick embryo or rabbit kidney cells overlaid with nutrient agar (Chaparas and Schlesinger, 1959; Foley and Remington, 1969; Akusawa, 1977). Our aim at present is to distinguish a susceptible cell from established cell line cultures such as HeLa (derived from human cervical cancer), BHK-21 (derived from baby hamster kidney), J-111 (derived from human leukemic leukocytes) and Peleg's *Aedes* mosquito (derived from *Aedes aegypti*) cells. Additionally, we aim to establish a reliable plaque assay for *Toxoplasma gondii* on this cellular substrate.

Materials and Methods

Toxoplasma gondii: Mouse-passaged *Toxoplasma gondii* (RH strain, kindly supplied by Dr. M. Iseki, Osaka City University School of Medicine) was employed. For serial intraperitoneal passages in dd mice, the *Toxoplasma gondii* was in the form of 20-fold diluted ascites in Eagle's MEM containing 10% calf serum. The passage was performed at regular intervals of 3-4 days from peritoneal exudate by injecting syringe.

Monolayer Tissue Culture: J-111 (established from human leukemic leucocytes; the Flow Laboratories, U. S. A. and the Dainippon Pharmaceutical Co., LTD., Japan), BHK-21 (established from baby hamster kidney cell), HeLa (established from human cervical cancer) and Peleg's *Aedes* mosquito cell lines (established from *Aedes aegypti*, kindly supplied by Dr. S. Hotta, of the Department of Microbiology in our School) were used for the experiments in *Toxoplasma* multiplication. Monolayer-cultured cells were prepared similar to those described previously (Hotta *et al.*, 1966; Shiraki and Hotta, 1977). The cells were seeded in plaque bottles for viral assay and incubated at 37 C until monolayers were obtained. The growth medium consisted of Eagle's MEM, 10% fetal calf serum (Gibco), $NaHCO_3$ (0.5% at a final concentration of 10% stock solution), supplemented with kanamycin 60 μg/ml at the final concentration. The maintenance media for the cultured cells were the same as the above-mentioned growth medium.

Footnote: Preliminary description of the studies was presented at the 33th West Regional Meeting of the Japanese Society of Parasitology, 16 October, 1977, in Kanazawa.

Methyl Cellulose Overlay Media and Plaque Assay: Methyl cellulose (abbreviated to *MC*) overlay medium was prepared by the following procedure, a modification of that proposed by Schulze and Schlesinger (1963).

MC solution:
MC 2 g
Distilled water 68 parts

Autoclaved at 120 C for 10 minutes and thereafter maintained at 45 C until mixed with Eagle's MEM solution.

MC overlay medium:
MC solution
70 parts (prepared as above-described)
Eagle's MEM (×5 concentrated)
20 parts
Fetal calf serum 10 parts
10% $NaHCO_3$ 0.5 parts

Mixed and shaked vigorously in ice water and stored until use.

After complete monolayers of J-111 cells were obtained in bottles, maintenance media were removed and the cells were inoculated with 4 ml of a suspension containing *Toxoplasma* in medium. The bottles were incubated at 39C for the indicated hours for adsorption (refer to RESULTS as shown in Fig. 4). After the medium was removed, the cells were washed with Eagle's MEM solution (pH 7.2) and 12 ml of the overlay medium were added to each bottle. The bottles were incubated at 39 C for 5 days. After the media were discarded, the cultures were stained with a solution of 0.04% crystal violet in alcohol including 0.8% ammonium oxalate to facilitate the counting of the plaques (Matsumura *et al.*, 1972). In order to compare the susceptibility of the cell line cultures employed to *Toxoplasma*, the number of the organisms released from cultured cells was counted under a light microscope (×400). At the same time, the morphological aspects of the multiplied organisms in these infected cells were also examined under a light microscope, by using Giemsa's staining method.

Results

Susceptibility of the Cultured Cells to Toxoplasma gondii:

J-111, HeLa, BHK-21 and *Aedes* mosquito cell cultures were used 24 hours after they were seeded in culture bottles. After inoculation with *Toxoplasma* (5×10^5 organisms), each cell culture was observed at the indicated intervals of days. In the case of HeLa cells, rossette formations of multiplied organisms in the cytoplasm could be observed one day after incubation and markedly increased in number 5 days after incubation, only resulting in the partial disruption of the cytoplasm. In these cultured cells, pseudocyst-like structures in the cytoplasm were also observed (Fig. 1a). In the case of BHK-21 cells, rossette formation of *Toxoplasma* could also be found one day after incubation. However, cellular destruction was not brought about and the multiplication of the organisms was less frequently found in the cytoplasm (Fig. 1b). In comparison with HeLa and BHK-21 cells, the rossette formation of *Toxoplasma* in J-111 cells and the cellular destruction induced by the growth of the organisms were clearly observed from one day after incubation (Fig. 1c). Five days after incubation, most of the culture cells were disrupted and numerous released organisms were observed around the degenerated cell nuclei. Although *Toxoplasma* attached to and penetrated *Aedes* mosquito cells 2 days after incubation at 29 C, any morphological changes of the infected cells and multiplication of the organisms could not be observed in further cultivation. Moreover, when the cells were cultivated under conditions varied from 29 C to 37 C, no trace of the multiplied organisms could be observed (Fig. 1d). The number of *Toxoplasma* organisms released from each cultured cell was counted at the indicated intervals of days and compared with each other as shown in Fig. 2. The number of released organisms in J-111 was much larger than that in HeLa cells. On the other hand, fewer organisms were released in the media of BHK-21 cells.

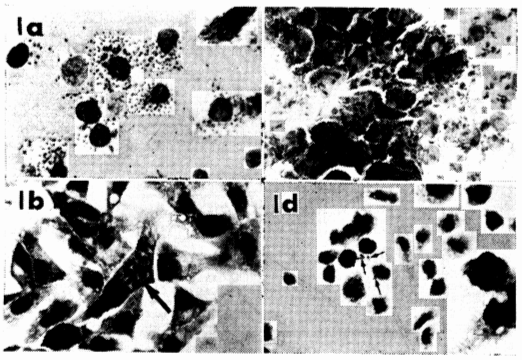

Fig. 1 Observation of the susceptibility of cultured cells to *Toxoplasma gondii*. Magnification ×800. (a) HeLa cells at 4 days after inoculation. In the cytoplasm of cells, the profiles of multiplication by binary fission and rossette formation of the organisms can be seen. (b) BHK-21 cells at one day after inoculation. Typical rossette formation of the organisms in the cytoplasm of the cells can be seen (arrow). (c) J-111 cells at one day after inoculation. Compared with the cases of HeLa (a) and BHK-21 (b) cells, multiplied organisms in the cells and their release to the intercellular spaces can be markedly observed. (d) *Aedes* mosquito cells at one day after inoculation. Although some organisms are seen in the cytoplasm of the cells, they do not multiply any more during further observation (arrows).

Plaque Formation by Toxoplasma gondii:

From the above-mentioned results, since J-111 cells appeared to be highly susceptible to *Toxoplasma*, plaque formation was tested by using cells from this culture, employing a modification of plaque assay for arboviruses as described elsewhere (Schulze and Schlesinger, 1963; Matsumura *et al.*, 1972). As shown in Fig. 3a, distinct round plaques, averaging approximately 1 to 2 mm in diameter were first visible on the 5th day after incubation. Numerous released organisms surrounding the degenerated cellular nucleus in one plaque were seen under a light microscope (Fig. 3b). The formation of plaques seen in these infected culture cells could not be found in any mock-infected culture cells. Plaques were not countable after 7 days incubation, because marked cellular degeneration occurred and the cells detached from the bottom of culture bottles.

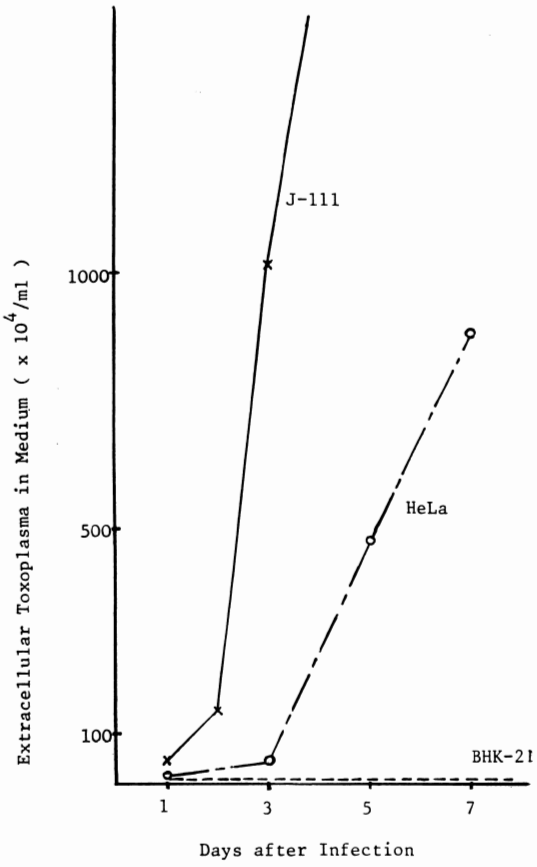

Fig. 2 Numbers of *Toxoplasma gondii* released from the culture cells, infected with 5×10^5 organisms. The data represent the numbers of the organisms released in the culture media of J-111 (×——×), HeLa (○- - -○), and BHK-21 (- - - -) cells, respectively.

Adsorption Time of Toxoplasma to Cells:

After a number of culture bottles were inoculated with *Toxoplasma*, duplicate bottles were taken at the indicated intervals (as shown in Fig. 4). The excess inoculum was removed as completely as possible and the overlay media were added. On the 5th or 6th day after the beginning of incubation at 39 C, the plaques which had been stained were counted as described in MATERIALS and METHODS. As shown in Fig. 4, the maximum plaque numbers were obtained in adsorption times of longer than 3 hours. Plaque numbers did not vary after the period

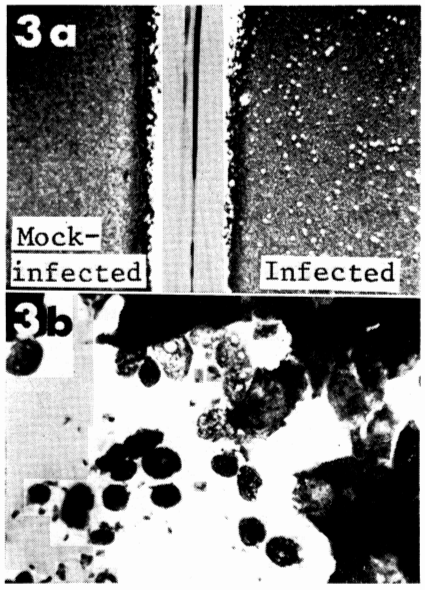

Fig. 3 (a) Plaques produced by *Toxoplasma gondii* (RH strain) on J-111 cell line culture in the plaque bottles. (b) Released organisms surrounding the disrupted cells in one plaque under the observation of a light microscope (Magnification ×800).

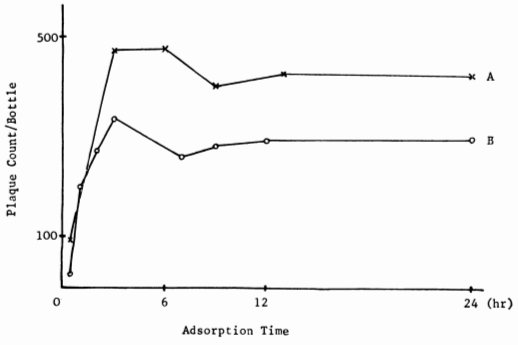

Fig. 4 Adsorption time of *Toxoplasma* to J-111 cell line culture for plaque formation. A and B indicate average number of plaques from the duplicate bottles infected with *Toxoplasma* organisms 1.4×10^3 and 6×10^2, respectively.

of 39 hours adsorption time.

Correlation of Plaque Count to the Number of Inoculated Organisms :

The correlation of the plaque count to the number of inoculated organisms between approximately 1,000 to 3,000 organisms as estimated directly by hemocytometer was

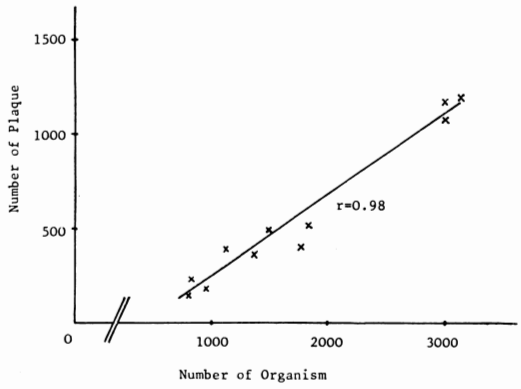

Fig. 5 The correlation of plaque count to the number of *Toxoplasma gondii*. Each sign indicates the number of plaques formed from one bottle.

demonstrated linearly (correlation coefficient r=0.98, Fig. 5). The efficiency of plating (EOP) calculated from the above-mentioned experiment was approximately 3.0 or 33% of the counted organisms.

Discussion

Although bioassay for viable *Toxoplasma* has already been studied in animals using mouse LD_{50} (Eyles and Coleman, 1956) and in mass tissue cultures such as LD_{50} in mouse embryo roller cultures (Chernin and Weller, 1957), the data described has been too variable to provide a suitable basis for quantitative studies of the organism itself. Thus, Chaparas and Schlesinger (1959) reported the first successful plaque formation by this organism, using agar-overlaid monolayers of chick embryo fibroblasts. Afterwards, Foley and Remington (1969) also reported that higher plaquing efficiency and reproducibility were accomplished by the use of secondary rather than primary cultures of chick embryo fibroblasts, also using the methods of agar-overlaid media.

In this study, the most stable and efficient plaque formation for *Toxoplasma* has been elucidated through the application of arbovirus plaque assay described previously. An established cell line culture derived from the human leukemic leucocytes (J-111) was

employed for this cellular substrate. The choice of this cell line was based on its marked release of *Toxoplasma* in Eagle's MEM medium cultures as follows (Fig. 2): (i) The J-111 cell is most susceptible to *Toxoplasma* multiplication, because it exhibits the highest number of released *Toxoplasma* in media and shows clearly cytopathic effect; (ii) Monolayerd J-111 cells are maintained after seeding for *Toxoplasma* multiplication under the methyl cellulose overlay media for 5 to 7 days cultivation. It was thought, therefore, that J-111 cells would adequately produce clear plaques of *Toxoplasma*. Methyl cellulose proposed elsewhere (Schulze and Schlesinger, 1963) as a solidifying ingredient of overlay media was confirmed to be satisfactory in the present work. Other technical specifics of plaque formation were defined as used in the case of chikungunya virus plaque assay except the incubation temperature at 39 C and the adsorption time for 3 hours, so that the results obtained were reproducible in repeated experiments.

The development of the *Toxoplasma* plaque was observed 5 to 6 days after incubation. The clearly-edged plaques, 1 to 2 mm in diameter, were formed in a countable manner. It was confirmed that the observation of *Toxoplasma* organisms and degenerated cellular debris in one plaque was due to a mechanism similar to the plaque formation produced by the infection of virus (Dulbecco and Vogt, 1954). The necessity of 5 to 7 days incubation to obtain clear plaques may be regarded as an advantage for biological experiments. Conditions earlier to detect *Toxoplasma* plaque formation are under study; e. g., the application of fluorescent antibody (Igarashi and Mantani, 1974) or immune-peroxidase (Okuno, 1978) technique. Basic phenomenon, such as the ratio of plaque count to inoculated *Toxoplasma* organisms were parallel and reproducible as shown in Fig. 5.

The results obtained so far offer promise that the plaque assay system described here is potentially useful for: (i) screening antitoxoplasma drugs (Dr. Y. Tsunematsu, personnal communication, 1977; Roberts *et al.*, 1976), (ii) selecting various *Toxoplasma gondii* clones (Pfefferkorn and Pfefferkorn, 1976), (iii) studying the unknown metabolism of the organisms (Pfefferkorn and Pfefferkorn, 1977), (iv) estimating antibody titer in serum, or (v) examing the changes of pathogenicity of tachyzoites, cysts and oocysts of the organisms in mammals (Ito *et al.*, 1976).

Summary

The susceptibility of various cell cultures to *Toxoplasma gondii* was studied in established cell line cultures such as J-111, HeLa, BHK-21 and Peleg's *Aedes aegypti*. Among them, J-111, HeLa and BHK-21 cells were susceptible, but mosquito cells not susceptible. Among susceptible cells, J-111 cell line infected with *Toxoplasma gondii* showed the most obvious cytopathic effect and released numerous organisms in the culture media.

By using the J-111 cell line, plaquing of *Toxoplasma gondii* was studied under a methyl cellulose overlay of Eagle's medium. Conditions such as the adsorption time of the organisms to cells (3 hours), incubation time (5 to 6 days) and incubatiton temperature (39 C) were defined to get reproducible results. The number of plaques under such conditions was reproducibly proportional to the number of the organisms inoculated.

Acknowledgements

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組織培養細胞における *Toxoplasma gondii* の研究
1. ヒト急性単球性白血病血液由来細胞（J-111）への感受性及びプラク形成

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Toxoplasma gondii の各種培養細胞に対する感受性を，J-111, BHK-21, HeLa, Peleg の *Aedes aegypti* の各培養細胞で検討した．このうち，J-111, HeLa, BHK-21 では感受性を認め得るが，蚊の細胞には認められなかつた．J-111 細胞は最も感受性が高く，著しい細胞変性が観察され，培養液中に多くの虫体放出が認められた．

この J-111 細胞を用い，*Toxoplasma gondii* のプラク形成を試みた．その至適条件は，吸着時間3時間，細胞培養5～6日間，培養温度 39 C で，重層培養液としてメチルセルローズ＋Eagle's MEM（10％牛胎児血清添加）を用いている．プラク数と接種虫体数とは，比例関係にあり，再現性が認められた．