Growth of Tyzzer's Organism in Primary Monolayer Cultures of Adult Mouse Hepatocytes

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The Tyzzer's disease organism was grown in primary monolayer cultures of adult mouse hepatocytes prepared by collagenase perfusion. The organisms produced a plaque-like cytopathic effect involving almost the whole culture around 72 h post-infection when the bacterial growth reached a maximum. The organisms showed specific immunofluorescence, and electron microscopy revealed that intracellular organisms had peritrichous flagella and underwent cell division. After intravenous inoculation of the infected cell culture into mice, necrotic hepatitis was produced and the organisms, recovered from the liver lesion, could be propagated in primary culture of mouse hepatocytes.

INTRODUCTION

Tyzzer's disease of laboratory mice is characterized by multiple focal necrosis in the liver and the presence of slender bacilli within hepatocytes (Tyzzer, 1917). The same disease has also been noticed in other species of laboratory, domestic and wild animals (Fujiwara, 1978). Because culture of the causative agent *in vitro* is still unattainable, information on its basic microbiology is lacking and its taxonomic position has remained undetermined. Successful propagation of the organism was described either in embryonic mouse tissue placed on agar slopes by Rights *et al.* (1947), or on agar medium containing mouse liver homogenate by Kanazawa & Imai (1959), but neither report was confirmed. Other than mouse passage, growth in chick embryos has been the only system available for propagating the organism (Fujiwara *et al.*, 1963; Craigie, 1966; Ganaway *et al.*, 1971 *a, b*). In order to elucidate the biological nature of the causative agent as well as its pathogenic mechanisms, *in vitro* culture of the organism would be of great value.

Recently, primary monolayer cultures of adult rat hepatocytes prepared by enzymic perfusion have been used to study hepatocyte physiology. The method was also adapted to the mouse to study hepatotropic virus infections (Arnheiter, 1980). The present report describes the successful growth of the Tyzzer's organism in a primary monolayer culture of adult mouse hepatocytes prepared by the same procedure.

METHODS

Animals. Female ICR mice (5 weeks old) obtained from a commercial breeder were used to prepare infective material and immune sera, and for pathogenicity tests. ICR mice (8–13 weeks old) from the same source were used to prepare hepatocyte cultures. The breeder colony had been proved by seromonitoring to be free from the main murine pathogens: Tyzzer's organism, *Corynebacterium kutscheri*, *Bordetella bronchiseptica*, *Mycoplasma pulmonis*, *Salmonella typhimurium*, murine hepatitis virus and Sendai virus (Fujiwara, 1971).

Hepatocyte cultures. Hepatocyte cultures were prepared by a modification of the methods described by Seglen (1976) and Arnheiter (1980). Mice were anaesthetized with Nembutal [50 mg (kg body weight)⁻¹] and the liver, *in situ*, was perfused via the portal vein with modified Hanks' solution free of Ca²⁺ and Mg²⁺ (37 °C) (Tanaka *et al.*,

Abbreviations: CPE, Cytopathic effect(s); FITC, fluorescein isothiocyanate.

1978) containing 0.5 mM-EGTA (Sigma), 10 mM-HEPES (Nakarai Chemicals, Kyoto) and 50 μ g kanamycin ml^{-1} (Meiji Seika, Tokyo), for 8 to 10 min at a flow-rate of 5 ml min^{-1} . Another perfusion was made for more than 20 min with the same solution without EGTA, containing 0.5 mM- CaCl_2 , 0.025% (w/v) collagenase (Type IV; Sigma) and 0.005% (w/v) trypsin inhibitor (Type II-S; Sigma). Perfused liver was sliced, and a crude cell suspension was made by gentle shaking and pipetting in culture medium (described below) held at 37 °C. After filtration through cotton gauze and then stainless mesh (no. 200), the suspension was chilled on ice and washed in the medium three times by centrifuging for 1.5 min at 30 g . The concentration of viable cells was adjusted to $5 \times 10^5 \text{ ml}^{-1}$ by trypan blue exclusion and 1.5 ml of the suspension was seeded into 35 mm dishes with or without glass coverslips, which were incubated at 37 °C in a humid atmosphere of 5% CO_2 in air. After 2 to 4 h, medium containing detached cells was aspirated and replaced with fresh culture medium.

The culture medium consisted of Williams' medium E (Flow Laboratories, Rockville, Md., U.S.A.) supplemented with 10% (v/v) calf serum (Flow Laboratories), 4 mU insulin ml^{-1} (Sigma), 1 μ M-dexamethasone (Sigma) and 50 μ g kanamycin ml^{-1} .

Organism. Rat-derived RT strain of Tyzzer's organism (Fujiwara *et al.*, 1971) was maintained in mice for more than 100 passages. Infected livers with multiple focal necrosis were obtained from mice which had been inoculated intravenously with infected liver homogenate after treatment with cortisone (Fujiwara *et al.*, 1964). Small pieces of liver were cut and stored at -80 °C. Just before inoculation, the livers were thawed and homogenized with the kanamycin-free medium. The number of organisms in the homogenate was determined by direct microscopic counting as described previously (Fujiwara, 1967), but modified by mixing the homogenate with an equal volume of phosphate-buffered saline containing 2% (v/v) formalin to minimize lysis of the organisms (Fujiwara, 1967).

Inoculation. After incubation of hepatocyte cultures for 20 to 30 h, when a monolayer was formed, medium was aspirated and each dish was inoculated with 0.5 ml of infective mouse liver homogenate containing 3×10^6 organisms in 1 ml kanamycin-free medium. After incubation at 37 °C for 2 or 24 h, culture medium was changed to 1.5 ml fresh kanamycin-free medium.

Observations. For macroscopic and microscopic observation, monolayers on culture dishes or on coverslips were fixed with methanol and stained with Giemsa or thionin.

The indirect immunofluorescence technique using FITC-labelled rabbit anti-mouse IgG has been described (Fujiwara *et al.*, 1969). To prepare antiserum against RT strain, mice received intranasal inoculation of freshly infected liver homogenate and, one month later, two subcutaneous injections 4 d apart with formalin-fixed homogenate. Animals were bled one week after the last injection.

For electron microscopy, coverslips, showing clear cytopathic effects (CPE) were fixed *in situ* with 2.5% (v/v) glutaraldehyde and then post-fixed with 1% (w/v) osmic acid. After embedding in Spurr resin (Taab Laboratories

Fig. 1. Cytopathic effects on mouse hepatocyte monolayers inoculated with RT strain of Tyzzer's organism. (a) 36 h, (b) 42 h, (c) 48 h, (d) 54 h, (e) 60 h and (f) 72 h post-infection. The monolayers were Giemsa-stained.

Fig. 2. Microscopy of Giemsa-stained plaque at 48 h post-infection. (a) Degenerated hepatocytes. The bar marker represents 100 μ m. (b) Abundant growth of organisms within living hepatocytes and some extracellular organisms at the margin of the same plaque shown in (a) (boxed). The bar marker represents 25 μ m.

Equipment, Reading, U.K.) and removing the coverslip, horizontal ultrathin sections of the monolayer culture were made and stained with uranium acetate and lead acetate. Specimens were examined using a JEM 100B electron microscope at 80 kV.

Growth kinetics. The number of organisms in cultures were estimated by counting of fixed, stained homogenates of whole culture or culture supernatant preparation.

Pathogenicity tests. After subcutaneous injection with 5 mg cortisone acetate (Cortone; Merck-Banyu, Tokyo), mice were challenged intravenously with 0.2 ml of homogenates of inoculated or uninoculated cultures. They were checked for mortality and liver lesions for 5 d post-inoculation.

RESULTS

Observations

Giemsa or thionin-stained preparations of inoculated hepatocyte cultures were shown to have weakly-stained spot-like plaques that could be observed macroscopically at 36 h post-infection (Fig. 1a). The plaques increased in size and number with incubation (Fig. 1b, c) fusing with each

Fig. 3. FITC-positive organisms within and at the margin of a plaque. Indirect immunofluorescence with anti-RT antibody. CPE are shown 48 h post-infection. The bar marker represents 100 μ m in (a) and 25 μ m in (b).

other at 50 to 60 h post-infection (Fig. 1*d, e*). Almost the whole surface of the culture was cleared around 72 h post-infection (Fig. 1*f*).

At 48 h post-infection, the CPE was seen to consist of degenerated cells with pyknotic nuclei (Fig. 2*a*). Within the cytoplasm of apparently viable hepatocytes surrounding this region, slender bacilli were present in bundles (Fig. 2*b*) as seen in infected mouse liver. Some apparently extracellular organisms were also recognized among degenerated cells. On prolonged incubation, even those cells not affected by CPE were seen to be parasitized. Few cells remained uninfected at 72 h post-infection.

The organisms in hepatocyte cultures were stained by indirect immunofluorescence using antibody to the RT organism. The CPE at 48 h post-infection consisted of an area of central necrosis with a number of scattered extracellular organisms that stained with specific fluorescence, and a surrounding layer of hepatocytes which fluoresced strongly (Fig. 3*a*). The organisms appeared to be located intracellularly and were arranged in parallel bundles occupying the cytoplasm, but not the nuclear region of hepatocytes (Fig. 3*b*). Electron microscopy revealed that the organisms with flagella resided within the hepatocyte cytoplasm and that some organisms were dividing within hepatocytes (Fig. 4).

Fig. 4. Electron micrograph of an organism undergoing cell division at 54 h post-infection. The bar marker represents 500 nm.

Fig. 5. Growth of RT strain of Tyzzer's organism in cultured mouse hepatocytes. Organisms (3×10^6 in 1.5 ml medium) were added to 7.5×10^5 hepatocytes per 35 mm dish and the medium was changed once at 24 h post-infection. ○, Counts in whole culture; ●, counts in culture supernatant.

Growth kinetics

A growth curve of the organisms in cultured hepatocytes was obtained by microscopic counting (Fig. 5). At 24 h post-infection, that is, just after the inoculum medium was changed, the number of organisms in the whole culture was only 10^3 organisms ml^{-1} . Later, organisms rapidly increased in number reaching a maximum around 72 h post-infection, and gradually declined thereafter. The number of organisms in culture supernatants was one third of that in the whole culture throughout the observation period.

Pathogenicity tests

The results obtained from the two experiments (I and II) are summarized in Table 1. While homogenates of the whole cultures sampled at 12 h post-infection or earlier had no effect, those sampled at 24 to 48 h post-infection were lethal for mice. The lethal effect decreased after 96 h

Table 1. *Pathogenicity for mice of RT strain of Tyzzer's organism grown in mouse hepatocyte culture*

Female ICR mice (4–5 weeks old) were challenged intravenously with 0.2 ml of homogenate of infected hepatocytes prepared by adding 3×10^6 organisms in 1.5 ml medium to 7.5×10^5 hepatocytes per 35 mm dish; medium was changed once 2 h (experiment I) or 24 h (experiment II) post-infection. Control animals received homogenate of uninfected hepatocytes.

Time after infection of hepatocytes (h)	Experiment I			Experiment II		
	No. of organisms (ml culture homogenate) ⁻¹	Mortality* of mice receiving:		No. of organisms (ml culture homogenate) ⁻¹	Mortality* of mice receiving:	
		Infected culture	Control culture		Infected culture	Control culture
2	4×10^4	0/5				
6	4×10^4	0/5				
12	8×10^3	0/5	0/5			
24	4×10^3	5/5		2.0×10^4	5/5	0/5
36	1.0×10^5	5/5		9.2×10^4	5/5	
48	8.4×10^5	5/5	0/5	1.5×10^6	5/5	0/5
72				5.4×10^6	5/5	0/5
84				6.2×10^6	5/5	
96				4.8×10^6	2/5	
120				2.7×10^6	0/5	0/5
144				8.4×10^5	0/5	

* Mortality is defined as (no. of dead mice)/(no. of tested mice) at 5 d post-infection.

post-infection; this correlated with a decline in the numbers of visible organisms. Affected mice showed multiple focal necrosis of the liver with the presence of organisms within hepatocytes. Organisms from the liver of affected mice could be cultured in hepatocytes. No liver lesions were produced in mice injected with uninoculated hepatocyte cultures.

DISCUSSION

The successful propagation of the Tyzzer's organism in tissue culture was described by Rights *et al.* (1947), who achieved abundant growth of the organisms in mouse embryo tissue placed on agar slopes. With chick embryo tissue, however, the results were not satisfactory. Craigie (1966) failed to infect a monolayer culture of the yolk sac endoderm from chick embryos, although some parasitized cells were seen when the culture was prepared from infected embryos. He attributed his failure to unsuccessful penetration of the organism into cultured cells rather than alteration in cell metabolism in culture.

In the present study, attempts were made to grow the Tyzzer's organism in a monolayer culture in order to obtain a convenient system for *in vitro* study. The criteria for *in vitro* propagation of the organism during the present studies were: (1) morphological changes in inoculated hepatocyte cultures, (2) presence of organisms in hepatocytes, (3) positive reaction of the organisms by immunofluorescence, (4) a progressive increase in bacterial concentration in infected hepatocytes and (5) production of the disease in mice inoculated with homogenates prepared from infected hepatocyte cultures. Since these criteria were fulfilled, our conclusion was that the Tyzzer's organisms actually grew in a monolayer culture of adult mouse hepatocytes. Since Tyzzer (1917), the organism has been known to be a strict intrahepatocytic parasite and the present study revealed that it appeared to grow within cytoplasm of cultured hepatocytes.

Both macroscopically and microscopically, CPE produced in the infected monolayer cultures showed characteristics similar to liver lesions seen *in vivo*. The organisms formed plaque-like CPE in monolayer cultures without agar overlay and the affected area was surrounded by infected hepatocytes. These findings strongly support the idea that cell-to-cell spread occurs *in vivo*. However, as the extent of CPE increased with prolonged incubation, it is probable that

organisms released extracellularly from damaged hepatocytes also infect other intact cells in culture. The present *in vitro* system might provide a great deal of information on cytopathogenicity of the organism.

The growth curve of organisms obtained in this study showed direct evidence of multiplication of the organisms in the culture. In view of the extreme fragility of these organisms in infected mouse liver homogenate (Fujiwara, 1967; Fujiwara *et al.*, 1973), the presence of 10^6 or more organisms ml^{-1} in the culture supernatant at 60 h post-infection or later can be considered as resulting from abundant growth of the organisms. In addition, the virulence of the propagated organisms for mice was demonstrated as early as 24 h post-infection as well as at 84 h post-infection. Such yield of infective organisms is of great advantage for preparing infective materials or specific antigens under controllable conditions compared to materials from infected mice.

Quantitative estimation of the organisms in experimental studies has been based upon direct microscopic counting of stained preparations (Fujiwara, 1967). By this method, however, it is impossible to determine the number of viable or infective organisms. Attempts were made to evaluate infectivity according to mean survival time of infected chick embryos (Craigie, 1966), 50% lethal dose for embryonated eggs (Ganaway *et al.*, 1971a), or mortality of inoculated mice (Fujiwara *et al.*, 1973). The present finding of CPE in inoculated monolayer cultures may provide another method of quantitative estimation by plaque assay.

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