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RED PLAQUE FORMATION OF *COXIELLA BURNETII* AND REDUCTION ASSAY BY MONOCLONAL ANTIBODIES

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Summary. — A red plaque technique for *C. burnetii* which utilizes primary chicken embryo cells, is described. Red plaques could be consistently detected as early as 6 days, usually 8 days post inoculation (p.i.), reflecting that *C. burnetii* proliferated within the phagolysosomes of host cells. Incubation with phase II monoclonal antibodies or inactivated immune sera containing phase I and phase II antibodies or phase II antibodies only, markedly reduced phase II *C. burnetii* red plaques. On the other hand, red plaques from phase I organisms increased several times when phase I cells were mixed with phase I monoclonal antibodies or inactivated immune sera containing phase I and phase II antibodies. By indirect red plaque reduction assay red plaque production by phase II cells could be reduced as well.

Key words: Coxiella burnetii; red plaque assay; red plaque reduction assay; monoclonal antibody

Introduction

Successful plaque formation by *C. burnetii* in primary chicken embryo cells has been reported by McDade and Gerone (1970), Wike *et al.* (1972), and Ormsbee and Peacock (1976). McDade and Gerone (1970) reported satisfactory results which were not confirmed by others. Wike *et al.* (1972) had difficulty in producing plaques by *C. burnetii* with an ordinary plaque procedure; modification of the procedure permitted the formation of plaques after 16 days of incubation at 34 °C. The plaques were indistinct and irregular in outline. Ormsbee *et al.* (1976) indicated that the use of neutral red had no effect on displaying plaques formed by *C. burnetii* after incubation at 36 °C for 13 days. Kordová (1966) and Cory *et al.* (1974) did not observe plaque formation by *C. burnetii* in primary chicken embryo and Vero cells, respectively. Since *C. burnetii* proliferates in the phagolysosomes, our objective was to develop an early detection method using the red plaque assay. We also studied the reduction of red plaques by monoclonal antibodies. The method seems useful for studying immunological properties of *C. burnetii* in tissue culture.

Materials and Methods

Rickettsial seeds. The *C. burnetii* Qiyi strain in phase I and the Grita strain in phase II were used in this study. The Qiyi strain was originally isolated from a patient of chronic Q fever. This strain was propagated in mice and chicken embryo yolk sacs (EP21/MP29). The Grita strain was passed 76 times in the yolk sacs of embryonated eggs in our laboratory. The previous passage history of this strain is unknown because it was obtained from the Soviet Union ir 1957. Seed stocks were prepared by homogenizing infected yolk sacs in sucrose-phosphate-glutamine (SPG) (Bovarnick *et al.*, 1950) to make a 10 % suspension, which was stored at −30 °C.

Cell culture. The procedure was a modification of that of Wike *et al.* (1972). Briefly, primary chicken embryo cells (CEC) were obtained from 10—11-day-old chicken embryos. Washed embryos were minced with scissors, and then placed in 50 ml Hanks' balanced salt solution at pH 7.6 containing sterile 0.25 % trypsin, digested for 30 min at 37 °C with stirring. The digest was filtered through six layers of sterile gauze, and centrifuged at 1500 rev/min for 10 min. The sedimented cells were resuspended in Medium 199 containing 5 % foetal calf serum, and five mililiter dispensed into each tissue culture at concentration of 10^6 CEC/cm^2. The flasks were incubated at 37 °C for 24 to 36 hr.

Red plaque procedure. After the monolayers were formed, the growth medium was decanted from the flask. Monolayers were washed once with Hanks' balanced salt solution. *C. burnetii* in 0.1 ml of serial 10-fold diluted inoculum was added to each CEC monolayer. The flasks were tilted immediately to insure rapid and even distribution of inoculum, and kept on a level surface for 1 hr at 34 °C. A nutrient overlay was prepared by mixing 2-fold concentrated Medium 199 at 37 °C with an equal volume of 1.8 % agarose in distilled water at 56 °C. The nutrient overlay (5 ml) was added to each flask after the mixture cooled to 45 °C. The tightly caped flask were incubated at either 32 °C, 34 °C or 36 °C. During the periods of incubation, the pH of overlay remained at about 6.8—7.4. The cell sheets were stained with 0.25 ml of 0.1 % neutral red solution prepared in Hanks' balanced salt solution. The red plaques were viewed on the indicated days.

Red plaque reduction assay. Ascitic fluids from hybridomas which secreted monoclonal antibodies against *C. burnetii* were diluted 100 times in Medium 199 containing 5 % foetal calf serum. Serial 10-fold dilution of polyclonal immune sera from guinea pig were prepared. Infected yolk sac suspensions containing 600—1000 PFU/ml for phase I *C. burnetii* and 3000—5000 PFU/ml for phase II organisms were mixed with equal volumes of ascitic fluids or inactivated immune sera, and incubated at 37 °C for 40 min. Monolayers were then inoculated with 0.1 ml of the mixture per flask, kept on a level surface for 1 hr at 34 °C, and then overlayed with the nutrient agarose medium. Normal mice ascitic fluids, normal guinea pig serum, ascitic fluids from hybridomas which secreted monoclonal antibodies against spotted fever rickettsiae, Jinghe strain, were used as controls.

Indirect red plaque reduction assay. The method of indirect red plaque reduction assay was carried out as follows. One half mililiter mixtures of ten times diluted ascitic fluids from B8, B5, and F1 hybridoma cells were dispensed into sterile tubes. Seeds of phase I *C. burnetii* Qiyi strain were diluted in Medium 199 containing 5 % calf serum. The final concentration of *C. burnetii* was about 1200 PFU/ml. One half mililiter of the diluted *C. burnetii* dispensed to each tube containing 0.5 ml of the various dilutions of monoclonal antibodies. The contents of the tubes were gently mixed and placed at 4 °C for 6 hr, and 0.5 ml of 1 : 5 diluted pig anti-mouse globulin was added to the tubes. After incubation at 34 °C for 40 min, 0.1 ml of the mixture containing rickettsial seeds, ascitic fluids of monoclonal antibodies, and pig anti-mouse globulin were dispensed into each flask.

Monoclonal antibodies against C. burnetii or spotted fever rickettsiae, Jinghe strain. Monoclonal antibodies directed against phase I or phase II *C. burnetii* were produced by Yu *et al.* (1986), titres are shown in Table 1. Hybridomas secreted monoclonal antibodies against spotted fever rickettsiae, Jinghe strain, were established in our laboratory, and the titre of the monoclonal antibody from one hybridoma used in this study was 1 : 64 000 by immunofluorescence test.

Immune sera. Antisera were obtained 14 days and 30 days after injecting guinea pigs intraperitoneally with 1 ml of ten times diluted suspensions of infected chicken embryo yolk sacs, distributed into tubes, and stored at −30 °C. These sera were titrated by the complement fixation (CF) test (Table 2).

Table 1. Titres of ascitic fluids as detected by ELISA

Ascitic fluids	Class and subclass	Antigen Phase I	Phase II
B5/I/	IgM	10^{-6}	10^{-1}
B8/I/	IgM	$\geq 10^{-10}$	10^{-1}
F1/I/	IgM	10^{-6}	10^{-1}
E5/II/	IgG_{2a}	10^{-2}	$\geq 10^{-10}$

Results

Red plaque formation by C. burnetii

After inoculation of the CEC monolayers with *C. burnetii*, the first change seen microscopically was the cytopathic effect (CPE) formation such as intracellular vacuoles in infected cells. CPE in host cells could be observed 3 days p.i. with seeds diluted 10^{-2} and 10^{-3}, and 6 days when seeds were diluted more than 10^{-4}. Initially, vacuoles were observed in only a few cells, and the size of the vacuole was small by microscopic examination at a magnification of 100 times. After 2 to 4 days the vacuoles were increased in size and the number of affected cells increased. The red plaques could be seen without the use of a microscope. After an additional 3 to 6 days of incubation, the red plaques were larger and more distinct (Fig. 1). The typical red plaques were 0.5 to 1.0 mm in diameter, distinct and irregular in outline. Under microscope the red plaques were composed of large numbers of vacuoles which stained deep red by neutral red (Fig. 2). After 16 to 18 days post inoculation the neutral red stain produced "colourless" plaques at the sites where the original early CPE was observed. Monolayers inoculated with normal suspensions of chicken embryo yolk sacs or diluent produced neither CPE nor red plaques. Cells obtained from red plaques were stained according to Gimenez, and numerous rickettsiae were observed.

There was a direct correlation between the dilution of inoculum and the number of red plaques (Table 3). Although the vacuoles of infected cells appeared about the same time by phase I and phase II organisms, there were some differences between them. Vacuoles produced by phase I organisms were larger and more regular in outline than those formed by phase II

Table 2. Titres of immune sera by CF test

Immune serum	Antigen Phase I	Phase II
14 days	1 : 8	1 : 1024
30 days	1 : 128	1 : 1024

Table 3. Red plaque counts on chicken embryo cells

Dilution of inoculum	Red plaque counts	
	Qiyi strain (phase I)	Grita strain (phase II)
10^{-6}	145.20 ± 16.54*	204.50 ± 29.13
10^{-7}	76.33 ± 5.74	47.67 ± 4.51
10^{-8}	26.67 ± 2.08	17.33 ± 1.53

* Mean number ± standard deviation ($\bar{X} \pm$ SD).

organisms. Red plaques of phase I organisms were 1.0 mm in diameter, irregular in outline, and distinct enough to be counted accurately on day 8 p.i. Phase II organisms produced somewhat indistinct red plaques after 10 days. Moreover, the typical size of red plaques was only 0.5 mm. Therefore, it was possible to differentiate phase I and phase II *C. burnetii* according to their vacuoles and red plaque morphology.

C. burnetii could produce red plaques at 32 °C, 34 °C, and 36 °C, but there were some differences in red plaques morphology. At 36 °C, red plaques produced by phase I organisms were smaller (0.5 mm) and to a certain extent indistinct after 10—12 days. Distinct and larger red plaques (1.0 mm) were formed by phase I cells both at 32 °C and 34 °C. Different incubation temperatures tested in our experiments did not cause significant differences in the occurence of red plaques.

Red plaque reduction assay by monoclonal antibodies

Phase II *C. burnetii* red plaques were reduced 81.6 % by monoclonal antibodies (ascitic fluids) from E5 hybridomas (phase II monoclonal antibodies,

Table 4. Effect of monoclonal antibodies on red plaque formation by phase I and phase II C. burnetii

Ascitic fluids from hybridoma of (10^{2} diluted)	Red plaque counts ($\bar{X} \pm$ SD)	
	Qiyi strain (phase I)	Grita strain (phase II)
B8(I)	138.00 ± 19.54	137.67 ± 18.77
B5(I)	63.75 ± 13.89	135.00 ± 15.90
F1(I)	86.00 ± 8.89	142.88 ± 13.20
E5(II)	38.75 ± 4.11	27.75 ± 7.41
Controls:		
Normal ascitic fluids	40.75 ± 3.86	143.75 ± 18.94
McAb SFR*	39.75 ± 6.45	151.00 ± 11.79
Diluent	40.50 ± 5.45	150.75 ± 16.28

* Monoclonal antibodies (ascitic fluids) against spotted fever rickettsiae, Jinghe strain.

Table 5. Indirect red plaque reduction assay of phase I C. burnetii

Experimental design	Red plague counts ($\bar{X} \pm SD$)
mixture of CbI[a], McAbI[b], and PAMIg[c]	24.33 ± 2.52
Controls:	
mixture of CbI, Dilu[d], and PAMIg	42.00 ± 2.83
mixture of CbI, McAbI, and Dilu	86.67 ± 9.71

[a] Phase I *C. burnetii*;
[b] Monoclonal antibodies against phase I cells;
[c] Pig anti-mouse globulin;
[d] Diluent;

McAbII), and were not affected by monoclonal antibodies from B8, B5, and F1 hybridomas (phase I monoclonal antibodies). On the other hand, phase I cells when mixed with monoclonal antibodies from B8, B5, and F1 hybridomas, their red plaques were enhanced 2 to 3 times as compared with either phase I cells treated with normal mouse ascitic fluids, or monoclonal antibodies against spotted fever rickettsiae Jinghe strain, or the diluent only (Table 4).

Because red plaques of phase I organisms could not be reduced by antibodies, we developed an indirect red plaque reduction assay. With this method, the red plaques produced by phase I cells were reduced by 42.1 %–71.9 % (Table 5). To confirm further the specificity of the above results, sera

Table 6. Effect of immune sera on plaque formation by phase I and phase II C. burnetii

Immune sera and their dilution	Red plaque counts ($\bar{X} \pm SD$)	
	Qiyi strain (phase I)	Grita strain (phase II)
early serum (14 days)		
1 : 10	39.50 ± 5.20	44.67 ± 13.05
1 : 100	38.00 ± 6.24	117.67 ± 11.50
late serum (30 days)		
1 : 10	177.50 ± 19.09	37.67 ± 5.51
1 : 100	81.67 ± 4.73	99.50 ± 20.62
1 : 1000	32.50 ± 2.12	149.33 ± 16.26
Controls:		
normal serum	33.75 ± 4.27	252.33 ± 22.90
diluent	38.67 ± 2.52	253.00 ± 16.70

containing phase I and phase II polyclonal antibodies, or sera containing only phase II polyclonal antibodies instead of monoclonal antibodies, were used in the red plaque reduction assay of *C. burnetii*. As shown in Table 6, the results obtained by immune sera were similar to that using monoclonal antibodies. Red plaques of phase II cells were reduced by sera containing phase II antibodies or phase I and phase II antibodies. The number of phase I red plaques had increased only times 4—5 by sera containing phase I and phase II antibodies when serum diluted 10-fold was used. Whereas, the number of plaques was not affected by the serum containing phase II antibodies only.

Discussion

There are basic differences between *C. burnetii* and other rickettsial species with respect to localization within host cells. All members of the genus Rickettsia grow within the cytoplasm of cells with no apparent association with vacuoles. *C. burnetii*, however, proliferates within vacuoles in the animal and in animal cells in culture (Baca and Paretsky, 1983). The vacuoles eventually fuse and form a single vacuole which occupies most of the cell's volume (Burton *et al.*, 1978). Cytochemical investigations revealed that the rickettsia-containing vacuoles are phagolysosomes (Burton *et al.*, 1971; Burton *et al.*, 1978), and their pH was about 5.1. Our results proposed that the formation of red plaques is due to acidic contents of vacuoles. When infected CEC monolayers were stained with neutral red, the intravacuolar neutral red appeared in a purple colour because of the acidity of vacuole content. Nevertheless, "colourless" plaques might appear only when infected cells were disrupted. Red plaque assay has many advantages: (1) It could be detected as early as 6 days, usually 8 days p.i. Whereas, "colourless" plaques needed 16—18 days of incubation in agarose overlay. (2) Appearance of red plaques was consistent. In our experiment, red plaques were formed every time, and their numbers were within the experimental error. But "colourless" plaques appeared occasionally, and were only formed in 17 of 38 experiments. (3) Red plaque morphology was used to characterize *C. burnetii* in phase I and phase II. Also, the "colourless" plaques were less evident and often could not be produced with precision.

The role of antibodies in immunity to rickettsial infection *in vivo* has been studied extensively. Most of the papers demonstrated that only immune sera containing antibodies to phase I antigen possess protecting or neutralizing capacity (Abrnanti and Marmion, 1957; Ormsbee *et al.*, 1e or ne *et al.*,n 1986). But *in vitro*, immune sera containing phase I antibodies were found to accelerate the entry of phase I organisms into the cells (Brezina and Kazár, 1965; Wisseman *et al.*, 1967; Kazár *et al.*, 1973). Our experimental result is consistent with the observation of Kazár *et al.* (1973). The red plaques produced by phase I organisms increased when phase I cells were mixed with phase I monoclonal antibodies which could protect mouse against *C. burnetii* infection (Yu *et al.*, 1986). We do not know how the explanation of this phenomenon indicating that although antibodies played a role in controlling Q fever, they also might promote infection by accelerating the entry of rickettsia into host cells.

Weinberg *et al.* (1969) and Wisseman *et al.* (1974) failed to develop plaque reduction assay of *Rickettsia rickettsii* and *Rickettsia prowazeki* in primary chicken embryo cells. Plaques of spotted fever rickettsiae were reduced only when indirect plaque reduction assay was used (Kenyon *et al.*, 1974), a result supporting our findings that phase I cells red plaques were reduced by indirect plaque reduction assay. Oaks *et al.* (1980) reported a successful plaque reduction assay of *Rickettsia tsutsugamushi* in L929 cells, and stating that rickettsial agglutination might play a role in plaque reduction. It remains a possibility that antibodies might prevent the entry of *Rickettsia tsutsugamushi* into host cells.

Our studies did not address the mechanism of red plaque reduction. Phase II *C. burnetii* easily auto-agglutinated suggesting that the agglutination of phase II cells induced by phase II antibodies might be the main mechanism of red plaque reduction. The explanation might also suit for indirect red plaque reduction assay of phase I organisms indicating the existence of infectious rickettsia-antibody complexes. Rickettsial viability would not be altered by agglutination but the apparent plaque titre would be reduced due to increased numbers of rickettsiae contributing to the formation of a single red plaque.

Red plaque assay could be used for isolation of *C. burnetii* directly from infected specimens. It is also a useful model for studying the interactions of *C. burnetii* and host cells.

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Cheng Xiao-xing, *et al.* (pp. 281—289)

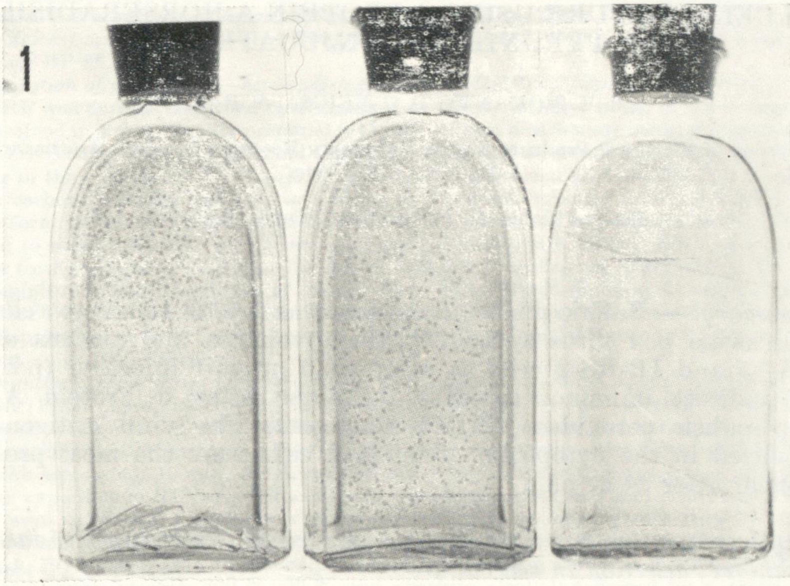

Fig. 1

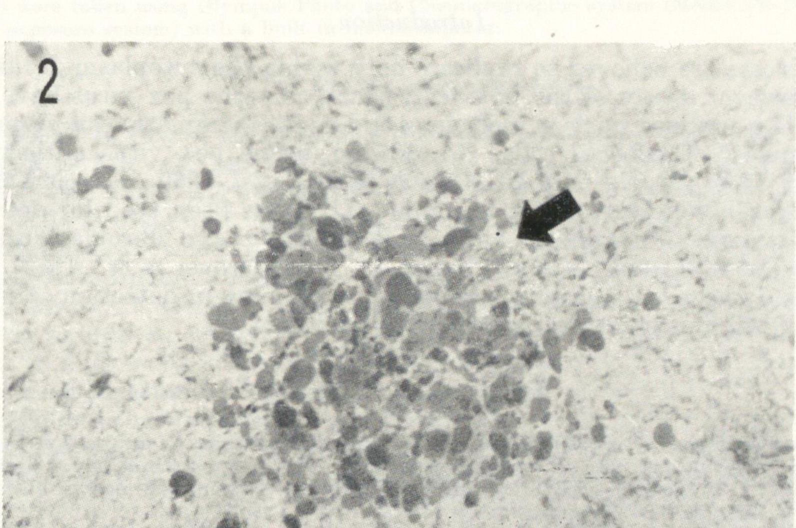

Fig. 2

Fig. 1. Red plaque formation of phase I *C. burnetii* in primary chick cell tissue culture at 12 days post inoculation. From left to right: 10^{-5}, and 10^{-6} dilutions of the Qiyi strain. At the right is an uninfected control.

Fig. 2. Under microscopic examination at a magnification of 100 times, the red plaques are composed of large numbers of vacuoles which stained deep red by neutral red.