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Studies of the Rickettsial Plaque Assay Technique

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A plaque assay system for pathogenic rickettsiae, which utilizes primary chick embryo tissue cultures, is described. It proved to be a highly reproducible measure of infectiousness for *Rickettsia rickettsi* and *R. typhi*, which were employed in most studies; as well as for *R. canada*, *R. prowazeki*, *R. sibirica*, *R. akari*, *R. conori*, and *Coxiella burneti*. Plaque-forming units (PFU) were compared to direct rickettsial counts and to 50% infectious dose (ID_{50}) values for embryonated eggs, mice, and guinea pigs. Plaque size, appearance, and number were influenced by diluent, incubation temperature after nutrient overlay, centrifugation of inoculated tissue cultures, and number of host cells planted initially in each flask. The most critical factors in plaque formation were diluent used in making rickettsial suspensions and incubation temperature (32 C) after nutrient overlay. Brain Heart Infusion was the only diluent capable of preventing significant delay in plaque formation and decreases in PFU and mouse ID_{50}. Plaque formation was unaffected by genetic background of host cells, volume of inoculum, temperature and length of incubation period before nutrient overlay, and rapid freezing and thawing of rickettsial seed. Centrifugation of inoculated cultures at 600 × *g* resulted in 100% irreversible absorption of rickettsiae to host cells within 5 min, whereas without centrifugation at least 4 hr was required to achieve the same effect.

Plaque formation by pathogenic rickettsiae in primary cultures of chick embryo cells has been reported by Kordova (8), Weinberg et al. (16), and McDade et al. (9, 10, 11). Weinberg et al. (16) also reported successful plaque formation by *Rickettsia rickettsi* in Vero cells, but failed to obtain plaques in hamster kidney, L, and cat kidney cell lines. Kordova had difficulty in reproducing her results with members of the spotted fever and typhus groups and failed to achieve plaque formation with *Coxiella burneti*. Weinberg and McDade were able to plaque most species of rickettsiae in a system similar, though not identical, to that of Kordova (8) and to elicit regularly plaque formation with good reproducibility of plaque numbers, although with considerable differences between infectious end points as judged by comparable titrations in animals or embryonated eggs.

Our studies were designed to identify factors which may influence plaque formation by rickettsiae in tissue culture, to improve the reliability and sensitivity of the plaque technique, and to describe some of the dynamic relationships between host cell and parasite which can be studied in tissue cultures. The technique of producing plaques employed in these studies was a modification of that of Weinberg et al. (16).

MATERIALS AND METHODS

Rickettsial seeds. Seeds were prepared by homogenizing infected yolk sacs in a blendor in a sucrose-phosphate solution (SP25) (18) to make a 50% yolk sac (w/v) suspension, which was dispensed in glass ampoules, rapidly frozen in a dry ice-95% ethanol bath, and immediately stored at −70 C.

The rickettsiae under study were *R. typhi* Wilmington strain, with a history of 12 embryonated egg, 15 guinea pig, and 2 embryonated egg passages (12EP/15GP/2EP); *R. rickettsi* Sheila Smith strain (10EP); *R. prowazeki* Cairo 3(17EP/1GP/1EP) and Breinl (162EP) strains; *R. canada* (8EP); *R. conori* Malish strain (10EP); *R. sibirica* (11EP); *R. akari* (9EP); and *C. burneti* Nine Mile strain in both phase I (306GP/2EP) and phase II (90EP).

Cell culture procedure. Chick embryo (CE) cell cultures were prepared from 10- to 11-day-old chick embryos. Embryos were decapitated, washed with medium 199 (M199; Grand Island Biological Co., Grand Island, N.Y.) containing 5% fetal calf serum (FCS; Grand Island Biological Co.) to remove red blood cells, and minced with scissors. The embryo mince was then placed in 100 ml of sterile 2.5% trypsin (Difco, 1:250) solution which had been adjusted to *p*H 7.8 with NaOH, digested at room temperature for 30 min with stirring, filtered through two layers of sterile cheesecloth (previously rinsed several times in hot distilled water), and centrifuged for 10 min at 150 × *g*. The sedimented cells were resuspended in M199 containing Hanks salts, L-glutamine, and 5% FCS and buffered at *p*H 7.5 with 7.5% sodium bicarbonate solution to provide a suspension containing 5 × 10^6 cells/ml (determined by hemocytometer count). Five milliliters of this suspension was then dispensed into each 30-ml (25-cm^2 surface area) plastic tissue culture flask (Falcon Plastics, Los Angeles, Calif.). Unless otherwise specified, 10^6 CE cells/cm^2 were planted in each

flask. The flasks were tightly capped and incubated at 36 C for 24 hr. No antibiotics were ever used in any tissue culture constituents.

Plaque procedure. The diluent for all inocula was ice-cold 3.7% Brain Heart Infusion broth (BHI, Difco) prepared by dissolving 37 g of dehydrated BHI in 1 liter of water unless otherwise specified. Also tested as diluents were SP25 and sucrose-phosphate-glutamate solution (SPG) (2). Rickettsial dilutions and suspensions were always held in crushed ice until inoculation. The fluid was decanted from each 24-hr confluent chick fibroblast monolayer, 0.1 ml of the inoculum was applied to each cell sheet, and the flasks were then immediately rocked at several angles to ensure distribution of the inoculum. The flasks were then placed on a flat surface and incubated at room temperature for 15 min. Five milliliters of nutrient overlay was then applied, and the flasks were closed immediately. Each 100 ml of nutrient overlay was prepared by adding 10 ml of distilled water to 0.5 g of agarose (SeaKem, Marine Colloids, Inc., Springfield, N.J.) and autoclaving at 20 psi for 15 min at 120 C. A 90-ml amount of the M199 with 5% FCS at 56 C was added to the melted agarose, and the mixture then was cooled to 45 C in a water bath just prior to overlay. After the overlay had gelled, the closed flasks were held at 32 C until plaques had developed.

After the plaques were well formed (9 or 10 days for spotted fever group rickettsiae, 12 or 13 days for typhus group rickettsiae), a final overlay of 5 ml containing neutral red was applied to each flask for 24 hr to increase plaque-cell sheet contrast before plaque counts were made. This overlay was prepared by adding 10 ml of 0.1% neutral red in 0.85% saline to 80 ml of M199 (buffered to *p*H 7.5, no calf serum), warming to 56 C, and adding to 10 ml of melted agarose. It was dispensed exactly as the primary overlay.

Plaque isolation technique. Before the neutral red overlay was applied, rickettsiae were isolated from plaques after first removing the upper side of the flask opposite the cell sheet with a hot wire. This was always done on the 8th day of incubation of the cell sheet for spotted fever group rickettsiae and on the 10th day for typhus group rickettsiae. A sterile, stainless-steel cork borer was then used to punch out an agar plug with attached cell sheet 4 mm in diameter. This plug was easily dispersed in ice-cold BHI with a syringe fitted with a 21-gauge needle. Such suspensions were then directly inoculated into tissue culture, animals, or eggs.

Rickettsial counts. A slight modification of the direct count method of Silberman and Fiset (14), originally developed for counting highly purified suspensions of *C. burneti* and chlamydiae, enabled us to obtain reproducible values for the number of rickettsiae in a crude yolk sac suspension. The modification involved overnight staining with acridine orange at a final concentration of 1:7500 and *p*H 5.5. Rickettsiae and bacteria were subsequently identified and counted in microdroplets by direct ultraviolet microscopic observation instead of in photographs.

Infectious end point determinations. The number of plaque-forming units (PFU) per milliliter was determined by averaging the plaque counts of three flasks at the dilution which gave between 30 and 80 plaques per flask for spotted fever group and between 50 and 150 plaques per flask for typhus or Q fever groups. The 50% infectious dose/ml (ID_{50}) values were determined for appropriate serial 10-fold dilutions of rickettsial seed by: (i) 1.0-ml intraperitoneal inoculation of groups of six 500-g Hartley guinea pigs; (ii) 0.5-ml intraperitoneal inoculation of groups of ten 12- to 14-g white mice (Rocky Mountain Laboratory strain); (iii) 0.5-ml inoculation of groups of 20 4- to 5-day-old embryonated chicken eggs which had been incubated at 36 C. Eggs inoculated with spotted fever group rickettsiae were incubated at 34 C; those inoculated with typhus group rickettsiae were incubated at 36 C. Inoculated eggs were candled daily. Yolk sacs of all eggs that died in the 12 days after inoculation were examined for rickettsiae by direct microscopic examination of tissue smears stained by the method of Gimenez (6). On the 13th day postinoculation (PI) the yolk sacs of remaining live and dead eggs were similarly examined. Yolk sacs of chick embryos still alive and negative by tissue smear on the 13th day were individually homogenized as 30% suspensions in BHI and inoculated into 10 eggs each which were similarly observed for visual evidence of rickettsial infection. Egg ID_{50} values were calculated from eggs found to be positive by tissue smear on the original titration or subculture, plus those eggs still alive on the 13th day PI and found to be negative by tissue smear on subculture. Eggs which died during the original 13-day period and which were found to be negative by smear examination were excluded from consideration. Groups of control embryonated eggs inoculated with BHI diluent alone showed spontaneous death rates of 26 to 100% by 13 days of incubation. Incubation at 34 C usually caused higher death rates than incubation at 36 C. Guinea pig and mouse ID_{50} values were determined by serologic conversion. Antibodies were measured by complement fixation (CF) (4) and microagglutination (MA) (5) tests with homologous antigens prepared by this laboratory. Tests were done on sera obtained 21 days PI. CF titers of $\geq 1:8$ or MA titers of $\geq 1:4$ were considered significant. ID_{50} values were computed by the Spearman-Kärber method (3).

Centrifugation procedure. Tissue culture flasks were inoculated with rickettsial suspensions and immediately placed upright in a modified International 1357A basket head (loaned for this study by Robert Gerloff of this laboratory) in an International centrifuge and centrifuged at 600 or 900 $\times$ *g* for 15 min. Room temperature was maintained in the centrifuge. The cell sheets then were immediately overlaid and incubated as usual.

RESULTS

Plaque morphology. Plaques formed by *R. typhi* (Fig. 1) were first detected 7 days after inoculation of the cell sheets, but the cultures required an additional 5 or 6 days of incubation before plaques were distinct enough to count accurately. They typically were 1.0 mm in diameter. This size and appearance also was characteristic of plaques produced by Cairo 3 and Breinl

strains of *R. prowazeki*. Plaques produced by *R. canada* also first appeared 7 days PI and required an additional period of 5 or 6 days for maximum definition, but typically were only 0.75 mm in diameter. Rapid nonspecific deterioration of the entire cell sheet often began after the 13th day of incubation. Plaques formed by *R. rickettsi* (Fig. 1) appeared 6 days after inoculation and required an additional 3 or 4 days of incubation before they were distinct enough to count accurately. The plaques typically were 2.0 mm in diameter. Other members of the spotted fever group rickettsiae, including *R. conori*, *R. sibirica*, and *R. akari*, produced plaques of size and appearance similar to those of *R. rickettsi*. In preliminary experiments, modification of tissue culture conditions, which included the use of an 8-ml nutrient overlay, an incubation temperature of 34 C, and *p*H 6.8, permitted the formation of plaques by *C. burneti*. These plaques averaged 0.75 mm in diameter, but only after 16 days of incubation. No differences in plaque morphology were observed between *C. burneti* phase I and phase II. Under these modified conditions, cell sheets resisted nonspecific deterioration for 18 days.

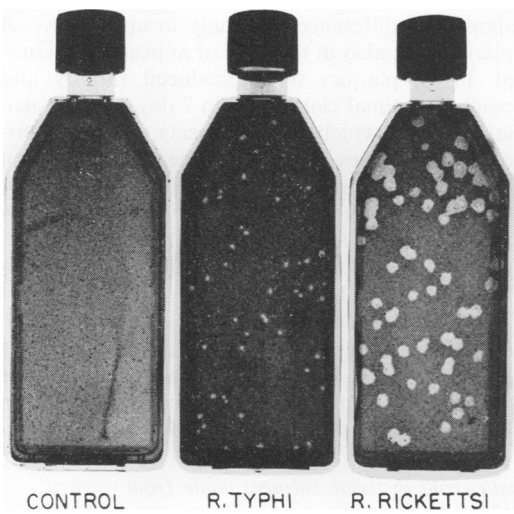

FIG. 1. *Rickettsial plaques in primary chick cell tissue culture. From left to right: uninfected control culture, R. typhi (Wilmington strain) plaques at 12 days postinoculation, and R. rickettsi (Sheila Smith strain) at 10 days postinoculation. Cell sheets stained with neutral red. Plaques are typical of typhus and spotted fever groups, respectively.*

PFU as a measure of infectiousness. With the number of morphologically identifiable rickettsiae in a given seed as a standard, we have compared PFU values for seeds of *R. typhi* and *R. rickettsi* with comparable ID_{50} values obtained in embryonated eggs, guinea pigs, and mice (Table 1). These data indicate that the PFU value for *R. typhi* was not significantly different from comparable ID_{50} values for embryonated eggs and mice, but was lower than the ID_{50} in guinea pigs by a factor of $\log_{10} = 0.9$. However, values in all bioassay systems were lower than the direct rickettsial count by 0.9 to 1.8 $\log_{10}$ units.

A different set of relationships was found for *R. rickettsi*. The PFU value was significantly higher than the corresponding mouse and embryonated egg ID_{50} values and higher, but not significantly, than the guinea pig ID_{50}. [Pickens et al. (12) have shown that mice inoculated with large concentrations of *R. rickettsi*, *R. conori*, or *R. sibirica* frequently produced little or no CF antibody response. Their sera were not tested for agglutinins.] The direct rickettsial count, however, was again significantly higher than any of the four bioassay systems. With this particular strain of *R. rickettsi* (Sheila Smith), the ID_{50} equals the 50% lethal dose (LD_{50}) in guinea pigs and eggs.

Variables which affected plaque number or morphology were as follows.

TABLE 1. *Comparison of direct rickettsial count with infectious end points in tissue culture, embryonated eggs, mice, and guinea pigs*

Seed	Titer ($\log_{10}$)[a] per g of yolk sac				
	Direct rickettsial count	PFU[b]	Egg ID_{50}[c]	Mouse ID_{50}	Guinea pig ID_{50}
Rickettsia typhi	11.0 (3)[d]	9.2 (3)	9.3 (1)	9.8 (3)	10.1 (1)
R. rickettsi	8.8 (1)	7.2 (3)	6.3 (1)	<3.8 (2)	6.7 (1)

[a] Standard errors of the means were as follows: direct rickettsial count $<\pm 0.1$, plaque-forming units (PFU) $<\pm 0.1$, egg $\pm$ 0.2, mouse $\pm$ 0.3, guinea pig ± 0.3.

[b] Plaque forming units.

[c] Fifty per cent infectious dose.

[d] Number in parentheses indicates the number of experiments done to determine the mean titer.

(i) **Numbers of cells employed in preparing tissue cultures.** Both the size and clarity of plaques were affected by the number but not the kinds of cells placed initially in the tissue culture flasks. If the decapitated embryos were not rinsed (in M199 with 5% FCS) to remove blood cells prior to mincing and tryptic digestion, approximately 50% (determined by hemocytometer count) of the final cell suspension used for implantation was composed of erythrocytes, which did not adhere to the surface of the tissue culture flask. The effects on plaque count of prior embryo rinsing in a typical experiment with *R. typhi* are presented in Table 2. These data indicate that flasks planted with cells from washed embryos $\geq 0.8 \times 10^6$ cells/cm^2 of flask surface or with cells from unwashed embryos $\geq 1.6 \times 10^6$ cells/cm^2 (or $\geq 0.8 \times 10^6$ adherent cells/cm^2) of flask surface yielded statistically indistinguishable plaque counts in their groups. However, with 1.2×10^6 cells/cm^2 (or 0.6×10^6 adherent cells/cm^2) from unwashed embryos, the plaque count had decreased by a statistically significant amount. It was noted also that the most sharply demarcated plaques characteristically were formed in flasks originally planted with washed embryo cells at 1.2×10^6 cells/cm^2 of flask surface or unwashed embryo cells at 2.0×10^6 cells/cm^2 (or 10^6 adherent cells/cm^2) of flask surface. The use of smaller numbers of cells in either category resulted in larger and less well defined plaques; the use of larger numbers of cells resulted in formation of smaller and much less differentiated plaques. Comparable results were obtained in similar experiments with *R. rickettsi*.

(ii) **Incubation temperature after agarose overlay.** The temperature range within which plaques were produced was relatively narrow. In the 8-degree temperature range (28 to 36 C) within which plaques were produced routinely, small temperature differences produced relatively large differences in size, appearance, and time of occurrence of plaques. Thus, *R. typhi* produced detectable plaques at 32 C, but not at 28 or 36 C. *R. rickettsi* produced plaques over the 28 to 36 C range, but statistically significant smaller numbers of them at 28 and 36 C than at 32 C ($P = 0.01$). The temperature of incubation also caused significant differences not only in appearance of plaques but also in the time of appearance. Thus, at 36 C, plaques were produced rapidly and reached optimal clarity within 7 days after inoculation, after which the cell sheets rapidly deteriorated. Plaques of the same size (2.0-mm diameter) were produced in 10 days at 32 C but were clearer and more sharply delineated than those produced at 36 C (Fig. 2). At 28 C, plaques were extremely small and difficult to count even 10 days PI or later.

(iii) **Diluents.** Diluents tested were BHI, SPG, and SP25 (all at *p*H 7.2). Results of plaque assays of *R. typhi* and *R. rickettsi* and parallel titrations of *R. typhi* in mice are shown in Table 3. In the plaque assay system, the seeds diluted with SPG

TABLE 2. *Comparison of plaque counts of Rickettsia typhi in tissue cultures made from washed and unwashed chick embryo*

Cell source	Chick embryo cells per cm^2				
	0.8×10^6	1.2×10^6	1.6×10^6	2.0×10^6	2.4×10^6
Washed embryos					
Count 1[a]	34	48	55		
2	37	54	74		
3	48	84	60		
4	57	45	49		
5		64			
Mean	44 (±11)[b]	59 (±16)	60 (±11)		
Unwashed embryos[c]					
Count 1		25	35	40	44
2		15	42	60	45
3		23	37	48	40
4		16	54	56	66
5			45		48
Mean		20 (±5)	43 (±8)	51 (±9)	49 (±10)

[a] Multiple values indicate replicate counts.
[b] Number in parentheses is standard deviation of the mean.
[c] Fifty per cent of these cells were chick embryo erythrocytes which did not adhere to the flask surface. Therefore, only half of the cells added actually formed the cell sheet.

and SP25 always underwent a sudden and marked drop in PFU before 0 time (15 min allowed for thawing and diluting the seed) when compared to seeds diluted in BHI. After the initial drop, the PFU values for seeds diluted in SPG or SP25 were relatively stable for at least 90 min if held at 0 C, except that SP25 was a less favorable holding medium than SPG for *R. typhi*, as indicated by the continued drop in PFU from 3.7×10^7 to 1.3×10^7 in SP25. The use of either SPG or SP25 diluents also resulted in decreased plaque diameter (by approximately 65%) and plaque clarity as illustrated in Fig. 3. Although the mouse ID_{50} of *R. typhi* was only slightly affected initially by use of SPG as compared to BHI diluent, the use of SP25 resulted in a dramatic decrease from 135×10^8 to 6.3×10^8 at 0 time. The continued deleterious effects of SPG and SP25 diluents after 0 time are demonstrated by comparison of the ID_{50} values for mice at 0 time and 90 min.

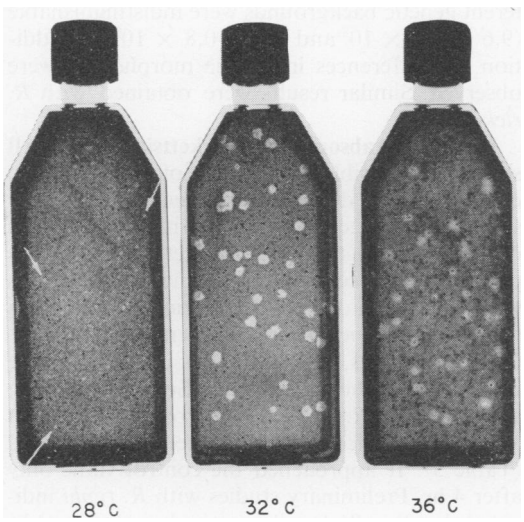

FIG. 2. *Effect of incubation temperature after agarose overlay on subsequent plaque development of R. rickettsi at 10^{-4} concentration of seed. Left to right: 28 C after 10 days of incubation, 32 C after 10 days of incubation, 36 C after 7 days of incubation.*

Results comparable with those for *R. typhi* were obtained for *R. prowazeki* Breinl in mice and tissue culture at 0 time (90 min not tested) with these three diluents, except that use of SPG did not prevent a marked drop in the mouse ID_{50}. The PFU values were 58×10^7, 2.7×10^7, and 1.3×10^7, and the mouse ID_{50} values were 31×10^6, 6.3×10^6, and 1.6×10^6 for BHI, SPG, and SP25, respectively.

(iv) **Centrifugation.** Centrifugation of inoculated cell sheets at $600 \times g$ for 15 min raised the average PFU for *R. typhi* by a factor of 5.9, and for *R. rickettsi* by a factor of 7.2 (Table 4). Centrifugation of *R. typhi*-infected cultures at $900 \times g$ did not raise the PFU significantly; at this and higher gravitational forces, the cell sheets were torn and subsequently underwent rapid degeneration. There was no observable effect of centrifugation at these gravitational forces on plaque size.

Variables which did not affect plaque number or morphology were as follows.

(i) **Freezing and thawing of rickettsial seed.** *R. rickettsi* seed, made as previously described except held at 4 C for 2 hr, was compared with identical samples shell-frozen at −70 C for the same time and then thawed. Statistically significant difference in PFU values was not observed, the values being 6.9×10^7 and 7.7×10^7 for frozen-thawed and fresh seed, respectively. Similar results were observed for *R. akari*.

TABLE 3. *Effect of various diluents on PFU[a] and mouse ID_{50}[b] values of Rickettsia typhi and R. rickettsi*

Seed	Diluent[c]	PFU		Mouse ID_{50}	
		0 min[d] at 0 C	90 min at 0 C	0 min at 0 C	90 min at 0 C
Rickettsia typhi	BHI	93×10^7	90×10^7	135×10^8	91×10^8
	SPG	6.0×10^7	5.5×10^7	91×10^8	2×10^8
	SP25	3.7×10^7	1.3×10^7	6.3×10^8	0.2×10^8
R. rickettsi	BHI	8.0×10^6	7.5×10^6	ND[e]	ND
	SPG	1.6×10^6	1.3×10^6	ND	ND
	SP25	1.4×10^6	1.2×10^6	ND	ND

[a] Plaque-forming units per milliliter.
[b] Fifty per cent infectious dose per milliliter.
[c] Abbreviations: BHI, Brain Heart Infusion broth; SPG, sucrose-phosphate-glutamate solution; SP25, sucrose-phosphate solution.
[d] Zero minutes equals the approximate 15 min to make dilutions and to inoculate immediately.
[e] Not done.

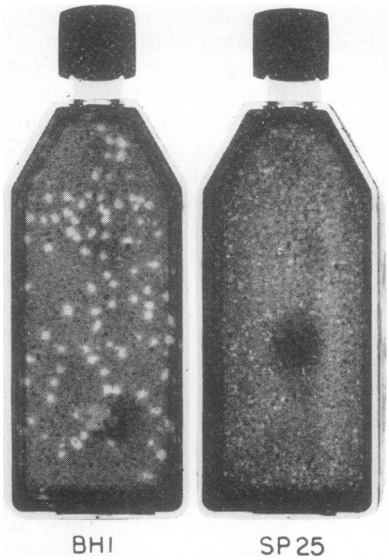

FIG. 3. *Effect of diluent on subsequent plaque diameter and clarity. R. typhi, 13 days postinoculation. Plaques from inoculum at 10^{-6} concentration of seed in Brain Heart Infusion (BHI) on left; plaques from inoculum at 10^{-4} concentration of same seed in sucrose-phosphate (SP25) on right. Effect of sucrose-phosphate-glutamate (SPG) solution on plaque morphology indistinguishable from that of SP25.*

TABLE 4. *Effect of centrifugation[a] on the number of plaque-forming units (PFU) per milliliter[b] of two rickettsial seeds*

Seed	Not centrifuged (PFU/ml)	Centrifuged 600 × *g* (PFU/ml)	Centrifuged 900 × *g* (PFU/ml)
R. typhi	9.6×10^8	57×10^8	63×10^8
R. rickettsi	3.0×10^6	22×10^6	ND[c]

[a] Fifteen minutes in International centrifuge in modified International 1357A basket head at room temperature.

[b] Determined by two separate experiments with three replicate determinations per experiment. Standard error of the mean <0.1 $\log_{10}$.

[c] Not done.

(ii) **Volume of rickettsial inoculum.** Three different volumes of BHI diluent, each containing the same number of *R. typhi* organisms per milliliter, were inoculated on CE cell sheets to determine the effect on plaquing efficiency. Varying the volume of inoculum over the range of 0.1 to 0.4 ml did not affect plaque count. Similar results were obtained with *R. rickettsi*.

(iii) **Temperature and length of incubation before overlay.** CE cell sheets were inoculated with *R. typhi* and incubated at 36 or 26 C for periods of 1 to 32 min and then were overlaid as usual and incubated at 32 C. Within the limits tested, neither temperature nor length of incubation before overlay significantly affected the eventual PFU values obtained. Similar results were obtained with *R. rickettsi*.

(iv) **Genetic background of chick embryo cells.** Eggs from two different flocks of chickens, one a Babcock Leghorn ♀ × Heisdorf-Nelson Leghorn ♂ cross and the other a Shaver Starcross 288 Leghorn ♀ × Welp California Grey ♂ cross, were used to make cell sheets which were then inoculated with *R. typhi*. Average PFU values in cultures of chick embryo cells from these different genetic backgrounds were indistinguishable ($9.6 \pm 0.7 \times 10^8$ and $9.5 \pm 0.8 \times 10^8$). In addition, no differences in plaque morphology were observed. Similar results were obtained with *R. rickettsi*.

Irreversible absorption of rickettsiae to CE cell sheets. Irreversible absorption of rickettsiae to cell sheets was defined by plaque production in spite of three successive washes with 2-ml volumes of BHI diluent to remove rickettsiae originally placed on the cell sheet. Inoculated cell sheets were washed as described at various intervals up to 4 hr after inoculation and then were overlaid. Control cultures, inoculated but unwashed, were overlaid at identical intervals. Some irreversible absorption of *R. rickettsi* occurred within 5 min and steadily increased over the period studied (Table 5). It approached the control value only after 4 hr. Preliminary studies with *R. typhi* indicated that this *Rickettsia* required more than 4 hr to effect irreversible absorption values approaching those of unwashed controls. On the other hand, centrifugation of cultures at 600 × *g* for 5, 10, or 15 min prior to washing caused complete irreversible absorption of both *R. rickettsi* and *R. typhi* to the cell sheet.

Clonal isolations. Isolation of rickettsiae from single plaques and subsequent culture in embryonated eggs or animals were uniformly successful, provided attempts were made prior to addition of the second (neutral red) overlay. Attempts to isolate rickettsiae from nonplaque areas of inoculated cell sheets were uniformly unsuccessful. These results were consistent with microscopic examinations of stained infected tissue cultures which revealed the presence of rickettsiae only within plaques and areas immediately adjacent to them.

DISCUSSION

Morphology of plaques produced by typhus and spotted fever group rickettsiae was found to be similar to that reported earlier (8, 9, 11, 16).

TABLE 5. *Time course of irreversible absorption of Rickettsia rickettsi to chick embryo cell sheets*

Cell sheet treatment	Incubation (min) before overlay or wash-overlay							
	5	10	15	30	60	120	180	240
Overlaid only..	72 (±9)[a]	67 (±3)	67 (±7)	65 (±6)	71 (±7)	64 (±10)	72 (±2)	77 (±8)
Washed,[b] then overlaid.....	12 (±3)	17 (±2)	21 (±3)	25 (±1)	29 (±4)	33 (±8)	50 (±4)	69 (±11)

[a] Mean plaque counts calculated from three replicate determinations. Value in parentheses is standard deviation of the mean.

[b] Three 2-ml washes with Brain Heart Infusion broth (at room temperature) per flask.

Plaques produced by members of the spotted fever group were larger, clearer, and more sharply delineated than those produced by the typhus group (including *R. canada*). Plaques produced by *C. burneti* were characteristically even smaller than those of the typhus group, indistinct, and irregular in outline.

The plaque assay technique was found to be the most sensitive measure of infectiousness for *R. rickettsi* and second only to the guinea pig for *R. typhi* under routine conditions. However, centrifugation of the inoculated tissue cultures before overlay increased the PFU by an average factor of 5.9 (Table 4) to a value indistinguishable from that for the guinea pig ID_{50} for *R. typhi*, and by an average factor of 7.2 to a value higher than that of any other bioassay system for *R. rickettsi*. Centrifugation has been shown similarly to increase the number of focal infections of chick embryo entodermal cells with *R. prowazeki* and *C. burneti* by Weiss and Dressler (17) and of L cells with *C. burneti* by Hahon and Cooke (7). Even centrifugation, however, did not bring PFU values of *R. typhi* and *R. rickettsi* up to their respective direct rickettsial counts. The reasons for the significant differences between direct rickettsial count and PFU values are unknown. It is possible that a significant fraction of the rickettsiae counted in the 50% yolk sac suspensions, which served as the infectious inocula, were dead. If so, they must have died before the infected yolk sacs were harvested, because neither freezing and thawing nor prolonged storage of seed at −70 C had a significant effect on PFU values. It is also possible that CE cells which constituted the tissue cultures varied in their capacity to support rickettsial growth and subsequent plaque formation. It may be significant that direct microscopic examination revealed, within plaque areas, the presence of CE cells which appeared entirely normal and uninfected.

The practice of overlaying tissue cultures 15 min after inoculation, when less than a third of the rickettsiae had become irreversibly attached to the cell sheet (Table 5), had little effect on the eventual numbers of plaques produced. Thus, many of the rickettsiae attached to the cell sheets well after the nutrient overlay was applied. However, if inoculated tissue cultures like those indicated in Table 5 were centrifuged at 600 × *g* for only 5 min instead of being left stationary, PFU values up to sevenfold greater were obtained. Thus, it is clear that even prolonged incubation of the inoculated cell sheet prior to overlay resulted in plaques being formed by only a fraction of the rickettsiae that were capable of doing so under the influence of centrifugation.

Kordova (8) reported that PFU values for typhus and spotted fever groups were 100-fold lower than corresponding LD_{50} values for embryonated eggs. Weinberg (16), however, reported that the PFU for *R. rickettsi* was indistinguishable from the egg LD_{50}. At the other extreme, McDade and Gerone (11) observed that the PFU was always greater than the egg LD_{50} for both spotted fever and typhus group rickettsiae. These discrepancies may be due to different sensitivities of the tissue culture systems in different laboratories. However, they may also be due to the fact that the egg LD_{50} is a very inaccurate measure of infectiousness, because, as we have reported, large numbers of embryonated eggs died spontaneously at 34 and 36 C when used as uninfected controls. The practice of excluding from our calculations those inoculated eggs which died during the 13-day period and were negative by smear may have tended to make the egg ID_{50} values slightly higher than they were in reality. But it did exclude that considerable percentage of embryonated eggs which died for irrelevant reasons, whose inclusion in ID_{50} calculation would have made these values unrealistically low. The reverse would be true, of course, if one were calculating an LD_{50}.

The number of CE cells used to form the cell sheets, regardless of whether erythrocytes were removed from embryos prior to mincing and tryptic digestion, did not affect the plaque count significantly, except at the lowest number tested (1.2×10^6 cells/cm^2 flask surface) for unwashed embryos (Table 2). These findings suggest not

only that a minimum cell density per square centimeter was necessary for efficient plaque formation, but also that the presence of chick erythrocytes neither contributed to nor interfered with eventual plaque formation. No evidence for interactions between rickettsiae and chick erythrocytes was apparent from these experiments.

Although *R. rickettsi* plaques were formed throughout the incubation temperature range tested, *R. typhi* produced plaques only at 32 C. The upper temperature limit for *R. typhi* plaque formation probably was associated with the failure of CE cell sheets to survive more than 7 days at 36 C; the lower limit probably was associated with inability of this normally small plaque producer to produce macroscopic plaques within the normal observation period of 13 days.

BHI was by far the best diluent for preparation of rickettsial suspensions. The use of either SP25 or SPG markedly reduced mouse ID_{50} and PFU values (Table 3), with the greatest reductions occurring in the 15-min period when dilutions of rickettsiae were actually being made. It is possible that the effect of SPG and SP25 on *R. rickettsi* was less because of the presence of a relatively greater amount of yolk and yolk sac in working suspensions of *R. rickettsi* (100-fold greater since *R. rickettsi* was used at 10^{-4} dilution, whereas both *R. typhi* and *R. prowazeki* were used at 10^{-6} dilution). Bovarnick et al. (2) have shown that small amounts (0.01%) of yolk sac partially protected against loss of infectiousness of *R. prowazeki* suspended in K7G (phosphate-buffered KCl plus glutamate). These workers also noted, as have we, that the deleterious effect of SPG on mouse ID_{50} was greater for *R. prowazeki* than for *R. typhi*. Bovarnick et al. (1) concluded that glutamate had a stabilizing effect on rickettsial suspensions. Our data do show that the drop in infectiousness is less in SPG than in SP25, but the difference between these two diluents in depressing infectiousness is much less (Table 3) than the difference between them and BHI. Rees and Weiss (13) suggested that *R. rickettsi* might produce toxic amounts of H_2O_2 from glutamate metabolism at low levels of oxygen tension. Our results do not indicate that this was a factor under the conditions tested because significant difference between *R. rickettsi* suspended in SPG or SP25 was not found.

Use of SPG and SP25 as diluents also decreased, the diameter of plaques by approximately 65% indicating that this diluent effect was a persistent one, and that those viable organisms which did eventually produce plaques may have sustained an insult which markedly increased the lag phase before infecting or multiplying, or both, in the CE cell sheets.

Our results suggest that published data on frequency of isolation from natural sources, as well as on apparent pathogenicity and metabolism of rickettsiae, may have been profoundly influenced by the diluent employed. Additional studies on the effects of a variety of media used in previous rickettsial studies are now underway in this laboratory.

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