Rickettsial Hemolysis: Rapid Method For Enumeration of Metabolically Active Typhus Rickettsiae

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A new assay is described for enumerating biologically active typhus rickettsiae (Madrid E strain), based on adsorption of rickettsiae to erythrocytes in the presence of NaF (which allows adsorption but not lysis) and lysis in the presence of anti-*Rickettsia prowazeki* immune serum (which allows only a single round of lysis). The number of lysed erythrocytes is then used to estimate the number of active rickettsiae.

There are two basic modes of rickettsial enumeration: (i) optical enumeration of total "rickettsia-like bodies" (RLB), and (ii) enumeration of "biologically active" rickettsiae. Whereas RLB determination is suitable for many types of studies, enumeration of biologically active rickettsiae is preferred for studies which depend on rickettsial activity. Relative numbers of biologically active rickettsiae can be enumerated by plaque formation on primary chicken embryo cultures (3, 8, 12), mouse, guinea pig, and egg 50% infectious dose determination (3, 8), and 50% fever dose determination in guinea pigs (3). However, these methods, if compared against each other, give widely divergent titers on an individual sample (3, 8). For example, Ormsbee et al. (3) reported that the titer of a *Rickettsia prowazeki* (Breinl strain) seed pool ranged from 0.3 to 67% of the RLB count, depending on the method of titration (calculated from data in Table 1, ref. 3). This indicates that these assays may measure different biological activities and that more than one viable rickettsia may be required to produce some of these measurable events (3). Thus, there is uncertainty as to which, if any, of these assays accurately enumerates (in an absolute sense) viable rickettsiae. Furthermore, the time frame required for plaque-forming unit or 50% infectious dose enumeration (3 to 21 days; 3, 8) is prohibitive for experiments that require discrete rickettsiae/ target cell ratios.

We have devised a rapid method to enumerate biologically active typhus rickettsiae based on their ability to lyse sheep erythrocytes under conditions where one rickettsia will lyse one erythrocyte. Using this technique, hemolytically active rickettsiae can be accurately enumerated in less than 90 min. This "antibody hemolysis" (AbHe) enumeration assay is based on the following: (i) at low ratios of rickettsiae to erythrocytes the rickettsiae distribute themselves among erythrocytes according to the Poisson distribution (7); (ii) rickettsial hemolysis, but not adsorption, is blocked in the presence of NaF (4, 10); (iii) in the presence of anti-rickettsial immune globulin, a rickettsia will lyse only the erythrocyte to which it is adsorbed (9); and (iv) only metabolically active rickettsiae will lyse erythrocytes (4).

Rickettsia prowazeki, Madrid E strain, was propagated in 6-day, embryonated, antibiotic-free hen's eggs by inoculation from a seed pool (seed pool passage no. 273). Rickettsial suspensions were prepared as previously described (2, 9, 11). Only fresh, unfrozen rickettsiae were used. RLB were enumerated by a modification of the method of Silberman and Fiset (6), i.e., directly rather than by photography. Sheep erythrocytes in Alsever solution were washed three times and suspended in sucrose-phosphate-glutamate solution (SPG; 1) at a concentration of approximately 10^{10}/ml. Erythrocytes were enumerated in a Fuchs-Rosenthal Ultra-plane counting chamber. The amount of hemoglobin released per erythrocyte when lysed in distilled water was determined by reading the absorbance at 545 nm (A_{545}).

The AbHe enumeration assay was initiated by adding rickettsiae (10^7 to 10^9 RLB in 0.2 ml of SPG) to sheep erythrocytes (5×10^9 in 0.4 ml of SPG containing 0.015 M NaF) and incubating for 30 min at 34°C. Incubation beyond 30 min did not result in increased rickettsial adsorption; maximum adsorption is complete in less than 5 min at 34°C (4). The suspensions were then diluted with 10 ml of ice-cold SPGMg (SPG containing 0.02 M MgCl$_2$) and centrifuged at 733 × g at 4°C. The pellet was suspended in 0.5 ml of SPGMg containing 10% (vol/vol) immune

serum from rabbits immunized with *R. prowazeki*, Madrid E strain (9). Anti-rickettsial serum has no effect on the lysis of preformed rickettsia-erythrocyte complexes, but agglutinates free rickettsiae and prevents their adsorption or readsorption (9). (This concentration of antiserum completely inhibited rickettsial adsorption when added to rickettsiae prior to the addition of erythrocytes.) The samples were then incubated for 30 min at 34°C to allow hemolysis to occur; hemolysis is complete in about 5 min at 34°C (4). At the end of incubation, 2.5 ml of 0.85% NaCl containing 0.08% formaldehyde was added, and the samples were centrifuged at 733 × *g* for 5 min at 4°C to pellet the erythrocytes. The number of erythrocytes lysed by the rickettsiae was determined from the A_{545} of this supernatant fluid and the A_{545} per lysed erythrocyte by using a standard curve.

When the ratio of rickettsiae to erythrocytes is 0.1 or below, according to the Poisson distribution (7), the number of lysed erythrocytes will approximate the number of active rickettsiae. For example, at a ratio of 0.1, rickettsiae will adsorb to 9.5% of the erythrocytes; 95.3% of these erythrocytes will possess only 1 rickettsia (5). If rickettsial distribution among erythrocytes is, indeed, Poisson, the number of hemolytically active rickettsiae can be estimated at ratios above 0.1 using the Poisson distribution to predict the number of erythrocytes with >1 adsorbed rickettsiae. Table 1 shows that enumeration of a rickettsial suspension over a 10-fold range of rickettsiae per erythrocyte was consistent, thus confirming that rickettsial distribution among erythrocytes was Poisson.

We enumerated rickettsial samples on 8 different days by both the AbHe method and the RLB method (6) (Table 2). Determinations were performed in duplicate, and three concentrations of rickettsiae were assayed in each experiment. Controls for spontaneous lysis were included, and these values were subtracted from the A_{545} readings. The AbHe enumerations ranged from 42 to 103% of the direct RLB count with a mean of 70% and a median of 58%. This indicates that, on most days, more than half of the rickettsiae were hemolytically active. This compares well with published reports (calculated from data in Table 1 of ref. 3) of 67 and 34% activity relative to RLB of two *R. prowazeki* seed pools assessed by guinea pig and L-cell 50% infectious dose determination, respectively. In contrast, it can be calculated that these same seed pools were only 4.5 and 1.5% active when assayed by plaque formation determination on chicken embryo fibroblast monolayers (3). These results may not be directly comparable with ours, however, as they enumerated rickettsiae which had been

TABLE 1. *Effect of variation of the rickettsia/ erythrocyte ratio on enumeration by the AbHe method*

Ratio[a]	% Multiple hits[b]	Active rickettsia ($\times 10^8$)
0.045	2.3	1.05
0.090	4.7	1.05
0.223	11.0	0.85
0.446	20.6	0.93

[a] A constant number of RLB (2.49×10^8) was added to a variable number of erythrocytes to alter the ratio. The ratio shown is the number of active rickettsiae per erythrocyte based on the 42% activity (1.05×10^8 active rickettsiae/2.49×10^8 RLB) found with the lowest ratio.

[b] The calculated percentage of all rickettsiae-erythrocyte adsorptions in which more than one rickettsiae would adsorb to an erythrocyte.

TABLE 2. *Enumeration of rickettsiae by the AbHe and RLB methods*

Rickettsiae ($\times 10^8$)[a]		Ratio: AbHe/RLB
AbHe[b]	RLB	
2.5 ± 0.0	5.9	0.42
1.1 ± 0.1	2.5	0.44
2.0 ± 0.2	3.8	0.53
4.5 ± 0.2	4.4	1.02
5.3 ± 0.0	5.9	0.90
2.2 ± 0.7	4.0	0.55
2.2 ± 0.1	3.8	0.58
6.0 ± 0.3	5.8	1.03
		0.68 ± 0.09[b]

[a] Individual paired determinations.

[b] Mean ± standard error.

frozen and thawed, whereas ours were freshly harvested. Furthermore, these low values for plaque formation can be increased 12-fold by centrifugation of the rickettsial inoculum onto the chicken embryo fibroblast monolayer (3).

Although the AbHe assay is a rapid and simple method to enumerate metabolically active rickettsiae, there are two limitations to the assay which must be noted. First, if an A_{545} of 0.025 above background is stipulated as the minimum absorbance required for significance, the lower limit of detection (sensitivity) is about 8×10^6 active rickettsiae ($0.025 A_{545} + 3 \times 10^{-9} A_{545}$ per erythrocyte). Second, the AbHe assay can be used to enumerate only hemolytically active rickettsial species. All typhus rickettsiae should be suitable, although only the Madrid E strain was investigated.

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