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Rickettsial Interactions with Human Endothelial Cells In Vitro: Adherence and Entry

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Rickettsia prowazekii, Madrid E strain, was assessed for its ability to enter endothelial cells derived from the veins of human umbilical cord in vitro. Rickettsial entry increased linearly with multiplicity of infection up to a multiplicity of 500; thereafter, additional rickettsiae adhered, but without a concomitant increase in the number of intracellular rickettsiae. Rickettsial entry required participation both of rickettsiae and endothelial cells; inactivation of rickettsiae with *N*-ethylmaleimide or Formalin, or of endothelial cells with cytochalasin B or D or NaF greatly reduced rickettsial entry. Because rickettsiae adhered to inactivated endothelial cells, adherence could be examined in the absence of entry. Rickettsial adherence was inhibited by poisons that inhibited rickettsial hemolysis. Calcium ionophore A23187, which did not inhibit endothelial pinocytosis, stimulated rickettsial adherence to endothelial cells, but inhibited rickettsial entry. These results indicated that typhus rickettsiae entered endothelial cells via induced phagocytosis, and that the signal for entry, which was dependent upon rickettsial energy, probably involved formation of a calcium gradient.

Rickettsia prowazekii is an obligate intracellular bacterium. Because *R. prowazekii* parasitizes a number of cell types in vitro, studies on rickettsial "penetration" into host cells have utilized such diverse model systems as erythrocytes (21–23, 37–39, 41), lymphoblasts (6), macrophage cell lines (28), and fibroblasts (6, 26, 32, 42, 44). At least three observations have consistently arisen from these reports. First, rickettsiae adhere specifically to the target cell before penetration. Wisseman et al. (44) reported that the presence of host cell fragments inhibited rickettsial penetration into fibroblasts. Ramm and Winkler (23), using rickettsial hemolysis as a model, reported that rickettsiae adhered to a cholesterol-containing site on the erythrocyte membrane as a necessary prelude to hemolysis. Walker and Winkler (32) reported that typhus rickettsiae adhered to inactivated L cells and that adherence was a saturable function of time and multiplicity of infection (MOI). Second, movement of adherent rickettsiae into a target cell requires active participation of such rickettsiae. Cohn et al. (6) and Walker and Winkler (32) reported that rickettsiae inactivated by any one of a variety of methods (heat, 2,4-dinitrophenol, UV irradiation, Formalin) were unable to enter target cells in vitro. Third, adherent rickettsiae cannot penetrate inactive target cells. Cohn et al. (6) reported that *Rickettsia tsutsugamushi* organisms were unable to penetrate heat-inactivated cultured mouse lymphoblasts. This observation was extended by Walker and Winkler (32), who reported that typhus rickettsiae adhered to, but did not penetrate into, fibroblasts that had been poisoned by treatment with thiol reagents (*N*-ethylmaleimide [NEM], p-chloromercuribenzenesulfonic acid), a glycolysis inhibitor (NaF), or an inhibitor of microfilament polymerization and glucose transport (cytochalasin B). These observations led Walker and Winkler to propose that typhus rickettsiae enter fibroblasts via induced phagocytosis. That is, rickettsiae adhere to a specific site on the target cell membrane and native, but not inactivated, rickettsiae perturb the target cell membrane to initiate phagocytosis at the sites of adherence. Phagocytosed rickettsiae then lyse the phagocytic vacuole and are released into the cytoplasm. Recently, Rikihisa and Ito (24) presented electron micrographic evidence that *R. tsutsugamushi* enters target cells by a similar process.

Unfortunately, none of the above studies examined interactions between typhus rickettsiae and their primary target cell—the endothelial cell (EC). This is important because EC differ markedly from fibroblasts. EC have been reported to (i) be rapidly pinocytic (7); (ii) express a number of unusual cell surface antigens such as factor VIII components (8, 12, 13), ABO antigens (15), and antithrombin III (4); and (iii) produce hemostatic effectors such as prostaglandin I_2, (3, 9, 19, 20), factor VIIIR:Ag (5, 12, 14), and factor $VIII_{VWF}$ (13). Thus, studies on how rickettsiae interact with EC are necessary not only to properly ascertain the specificity and mode of rickettsial entry into their primary target cell, but also to determine the molecular pathogenesis of the hemostatic and thrombotic perturbations which characterize typhus and spotted fevers.

We examined penetration of *R. prowazekii* into human unbilical cord vein-derived EC by using the radioactive technique of Walker and Winkler (32). Our studies suggest that typhus rickettsiae enter EC via induced phagocytosis.

MATERIALS AND METHODS

Rickettsial growth and preparation. *R. prowazekii*, Madrid E strain, was propagated in 6-day embryonated, antibiotic-free hen eggs by inoculation from a seed pool (yolk sac passage no. 272). Rickettsial suspensions were prepared from heavily infected yolk sacs by a modification (37) of the methods of Bovarnick and Snyder (2) and Wisseman et al. (43). Rickettsiae were used immediately after being harvested or were frozen at −80°C for later use. Viability of prepared rickettsiae was monitored by a modification (21–23) of the hemolytic procedure of Snyder et al. (27) and by the antibody hemolysis method of Walker and Winkler (33). Rickettsiae were enumerated by a modification (32) of the method of Silberman and Fiset (25), and the number of viable rickettsiae was determined by a modification of the method of Walker and Winkler (33). Because some rickettsi-

ae lost viability upon freezing and thawing, most results were expressed as a function of the number of hemolytically active rickettsiae.

The diluent for rickettsial suspension in the purification procedure was a sucrose-phosphate-glutamate solution described by Bovarnick et al. (1). The diluent for experiments was sucrose-phosphate-glutamate containing 0.01 M $MgCl_2$ and 0.1% D-glucose (32).

Radioactively labeled rickettsiae were prepared by incubation of rickettsiae with [α-^{32}P]ATP as previously described (32, 36). Labeled rickettsiae contained approximately 3.6×10^{-3} cpm per rickettsia. Rickettsial suspensions were washed to remove extracellular [α-^{32}P]ATP and suspended at appropriate concentrations.

Cell culture. EC were derived from human umbilical cord veins by a modification of the method of Jaffe et al. (15). Briefly, human umbilical cords were obtained immediately after normal vaginal deliveries and were stored until use at 4°C in 0.01 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.4), which contained 0.14 M NaCl, 0.004 M KCl, and 0.01 M D-glucose. Because cord viability dropped precipitously after 32 h, cords were used within 30 h postpartum. EC were released from the subintima by treating the cord vein for 10 min at 37°C with a 0.1% collagenase solution (in HEPES buffer). The cells were washed, counted in a hemacytometer, and suspended at the desired concentrations. Flasks (25 cm^2) were seeded with approximately 3×10^6 cord vein-derived cells and incubated at 37°C until confluent; the cells were then transferred to 30-mm culture dishes. EC were identified by their typical "cobblestone" appearance (8, 15), strict contact inhibition (15, 16), and production of prostacyclin (3, 9, 19, 20) and factor VIIIR:Ag (12–14, 16). Prostacyclin production was determined by radioimmunoassay, and factor VIIIR:Ag was identified by immunofluorescence (16). EC were cultured in medium 199 (containing Hanks salts) supplemented with 200 μM L-glutamine, 20% heat-inactivated fetal calf serum, 20 mM HEPES, and 320 μg of EC growth supplement per ml; EC growth supplement was derived from bovine neural tissue by the method of Maciag et al. (17, 18). Only confluent monolayers were used for experiments.

Some experiments utilized fibroblasts (L cells or BSC-1). Fibroblasts were cultured in medium 199 (containing Hanks salts) supplemented with 10% calf serum.

All tissue culture flasks and dishes were treated with 0.2% gelatin for 24 h before being seeded with either EC or fibroblasts.

Rickettsial adherence and penetration. Rickettsiae labeled with [α-^{32}P]ATP were suspended in sucrose-phosphate-glutamate containing 0.01 M $MgCl_2$ and 0.1% D-glucose (1.0 ml) and were incubated with EC or fibroblast monolayers in 30-mm culture dishes; the number of adherent and intracellular rickettsiae was determined as previously described (33, 34). Briefly, [α-^{32}P]ATP-labeled rickettsiae were incubated with target cell monolayers for 15, 30, or 60 min at 37°C. To determine the total number of target cell-associated rickettsiae, the supernatant was carefully removed, and extracellular nonadherent rickettsiae were removed by gently washing the monolayer five times with warm (37°C) phosphate-buffered saline. The monolayer was then extracted with 2% Triton X-100, a sample was mixed with Aquasol in a scintillation vial, and radioactivity was determined in a Beckman LS-100 liquid spectrometer. The number of rickettsiae associated with the monolayer was determined as previously described (32, 34). This calculation took into account the number of rickettsiae and target cells present, the radioactivity per rickettsia, the loss of radioactivity from rickettsiae during incubation, and the accumulation of labeled material from the incubation medium by the target cells. The number of intracellular rickettsiae was determined as described above, except that, 2 min before termination of incubation, excess unlabeled ATP was added to the incubation medium to chase the unlabeled ATP from extracellular rickettsiae. As reported earlier (32), this resulted in loss of >90% of the label from extracellular rickettsiae within 60 s. The number of adherent rickettsiae was then determined by subtracting the number of intracellular rickettsiae (chased sample) from the total number of target cell-associated rickettsia (unchased sample). Results were expressed as the number of rickettsiae per cell.

Rickettsial treatments. Rickettsiae were inactivated by treatment with 3% formaldehyde, or by incubation with 1 mM NEM at 37°C; unreacted NEM was neutralized by the addition of 10 mM 2-mercaptoethanol. Other treatments included 10 μm nonactin, 25 μM calcium ionophore A23187, and 40 mM NaF. The ability of treated rickettsiae to lyse sheep erythrocytes was used as an indicator of rickettsial activity (21, 22, 37, 38).

EC treatments. EC were inactivated by treatment with 1 μM NEM, 40 mM NaF, or 10 μg of cytochalasin B or D per ml. Unreacted NEM was neutralized as described above. The ability to pinocytose [*U*-^{14}C]sucrose and to transport [4,5-^{3}H]leucine was used as a check on the relative metabolic state of treated EC.

Materials. [α-^{32}P]ATP, [*U*-^{14}C]sucrose, and [4,5-^{3}H]leucine were obtained from ICN Chemical and Radioisotope Division. Collagenase (type I) was obtained from Worthington Diagnostic Systems, Inc. Other chemicals and their sources were as follows: NEM, 2-mercaptoethanol, atractyloside, ATP, cytochalasins B and D, nonactin, A23187, and HEPES, Sigma Chemical Co.; NaF, Mallinkrodt; Triton X-100, New England Nuclear Corp.; formaldehyde, Fisher Scientific Co. Bovine neural tissue was obtained from Pel-Freez, Inc. Some endothelial growth supplement was provided by Thomas Maciag, Beth-Israel Hospital, Boston, Mass.

RESULTS

Rickettsial association with EC. Typhus rickettsiae were incubated with EC monolayers at various MOI for 60 min, and the number of adherent and intracellular rickettsiae was determined as described above. Figure 1 indicates that the number of intracellular rickettsiae was a linear function (r = 0.950) of MOI up to an MOI of 250; thereafter, there was little apparent increase in the number of intracellular rickettsiae. The total number of EC-associated rickettsiae, however, continued to increase with MOI up to an MOI of 1000. As Fig. 1 shows, this additional increase was due to adherence with little additional rickettsial penetration. Preliminary evidence indicated (data not presented) that the reduction in EC-associated rickettsiae seen at the highest MOI was accompanied by EC damage and leakage of EC cytoplasmic material. *R. prowazekii* was previously reported to damage or destroy erythrocytes (21–23, 37–39, 41), L cells (40), and rabbit polymorphonuclear leukocytes (34).

Because *R. prowazekii* infects primarily EC in vivo (31), rickettsiae were incubated with EC or fibroblasts (BSC-1, L cells) at an MOI of 500, and the number of target cell-associated rickettsiae was determined. Figure 2 shows that rickettsiae did not associate with or enter EC more rapidly than they did fibroblasts. Thus, the preferential infection of EC by rickettsiae in vivo is not likely to be due to unusual

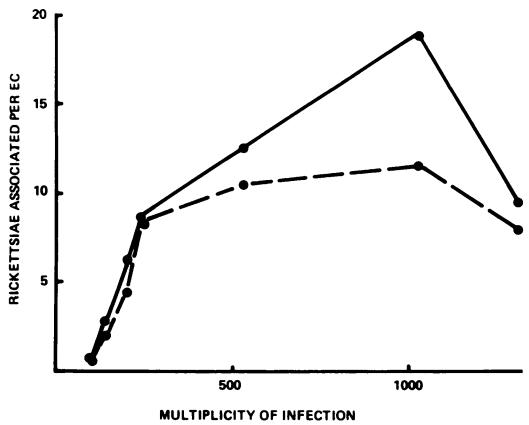

FIG. 1. Relationship of rickettsial association with EC to the MOI. Rickettsiae were incubated with EC at various MOI (rickettsiae per EC) for 60 min at 37°C, and the number of EC-associated rickettsiae was determined as described in the text. The solid line indicates the total number of EC-associated rickettsiae, and the hatched line indicates the number of intracellular rickettsiae.

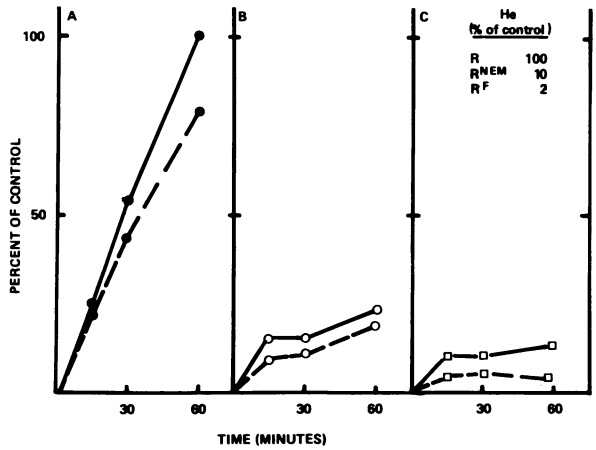

FIG. 3. Association of hemolytically inactive rickettsiae with EC. Native (A), NEM-treated (B), or Formalin-treated (C) rickettsiae at an MOI of 500 were incubated with native EC, and the number of EC-associated rickettsiae was determined as described in the text. The relative ability of treated and untreated rickettsiae to lyse sheep erythrocyts (C) was determined to assess rickettsial viability. EC association results are expressed as the percentage of the total number of native rickettsiae that associated with EC at 60 min. Solid lines indicate total EC-associated rickettsiae, and hatched lines indicate the number of intracellular rickettsiae.

susceptibility of EC to rickettsial adherence and entry. Figure 2 also shows that rickettsial adherence and entry into EC increased linearly ($r = 0.998$) for 60 min at 37°C. At each time point most of the EC-associated rickettsiae were intracellular. This indicated that, as reported earlier for L cells (32), adherence was the rate-limiting step in the rickettsial entry process.

Dependence of entry on rickettsial activity. Because earlier studies (6, 32) indicated that rickettsial activity was required for entry into other cell types, rickettsiae were inactivated by treatment with NEM or formaldehyde and were assessed for their ability to enter EC (Fig. 3). Rickettsial entry by inactivated rickettsiae was significantly reduced relative to that of their viable counterparts. NEM or Formalin treatment of rickettsiae, which reduced rickettsial hemolysis to nearly zero, inhibited rickettsial entry during 60 min of incubation by 76 and 95%, respectively. This indicated that the process by which rickettsiae entered into EC was dependent, at least in part, upon rickettsial energy.

Dependence of entry on EC activity. Because Walker and Winkler (32) reported that rickettsial entry into L cells required both rickettsial and L-cell activity, EC were treated with the phagocytic inhibitors cytochalasin B, cytochalasin

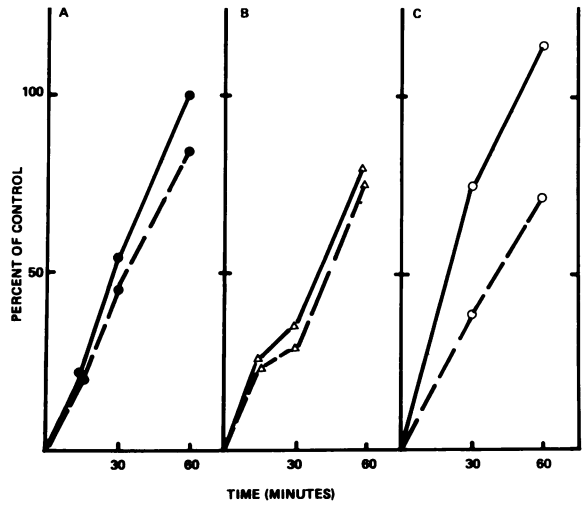

FIG. 2. Association of *R. prowazekii* with three types of target cells. Rickettsiae were incubated at an MOI of 500 at 37°C with (A) EC, (B) L cells, or (C) BSC-1 fibroblasts as described in the text. Solid lines indicate the total number of target cell-associated rickettsiae, and hatched lines indicate the number of intracellular rickettsiae. Results are expressed as the percentage of the total number of EC-associated rickettsiae at 60 min.

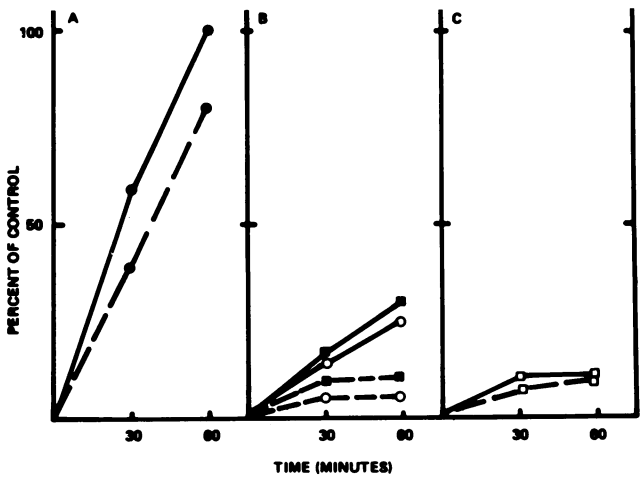

FIG. 4. Effect of EC treatments on rickettsial association with EC. Native rickettsiae were incubated with (A) untreated EC (●), or EC that had been treated with (B) NEM (○) or cytochalasin D (■). Some rickettsiae were incubated with EC in the presence of (C) NaF (□). Solid lines indicate total EC-associated rickettsiae, and hatched lines indicate intracellular rickettsiae. Results are expressed as the percentage of the total number of rickettsiae associated with native EC after 60 min at 37°C and an MOI of 500.

TABLE 1. Effects of treatments on rickettsial and EC activities[a]

Treatment	Rickettsial activities		EC activities		
	Hemolysis	EC adherence	Pinocytosis	Leu[b]	Rickettsial entry
None	100	100	100	100	100
NaF (40 mM)	0.9	54	17	44	11
NA (10 μM)	49	65	96	97	ND[c]
A23187 (25 μM)	5	272	100	59	28
R^{NEM}	0.4	28	ND	ND	24
R^{F}	0.0	43	ND	ND	6

[a] NA, nonactin; A23187, calcium ionophore A23187; R^{NEM}, NEM-treated rickettsiae; R^{F}, Formalin-treated rickettsiae. All results are expressed as percentages of the untreated control.

[b] Leucine transport.

[c] ND, Not determined.

D, NEM, or NaF and were then incubated with native rickettsiae. As Fig. 4 shows, each of these inhibitors halted rickettsial entry into EC; results obtained with cytochalasin B (data not shown) were identical to those obtained with cytochalasin D. These results indicated that rickettsial entry into EC was dependent upon EC activity, probably phagocytosis. Furthermore, because rickettsiae adhered to NEM- and cytochalasin-treated EC, this provided a convenient means to examine rickettsial adherence in the absence of entry. As Fig. 4 shows, however, rickettsiae neither entered nor adhered normally in the presence of NaF; this suggested that NaF interfered with both processes.

Examination of the mechanisms of rickettsial adherence and penetration. Rickettsiae were incubated with native or NEM-inactivated EC in the presence of NaF, nonactin, or calcium ionophore A23187, and adherence and entry were determined. EC pinocytosis and leucine transport were monitored to determine the effects of the aforementioned treatments on native EC activities. Table 1 shows that NaF, which blocked rickettsial hemolysis, inhibited both rickettsial adherence to the target cell and subsequent entry. The potent inhibitory effect of NaF on entry was paralleled by a marked inhibition of EC pinocytic activity. Nonactin, which acts as a membrane K^+ pore (10), inhibited hemolytic activity by approximately 50% and inhibited rickettsial adherence to EC by 35%; measured EC activities were not inhibited. Calcium ionophore A23187, which has an effect similar to that of a membrane calcium pore (3), reduced rickettsial hemolysis by 95% and did not affect EC pinocytosis. Yet, A23187 increased rickettsial adherence to EC significantly and inhibited rickettsial entry into EC by 72%. This suggested that rickettsial entry, but not adherence, required that rickettsiae or EC form a Ca^{2+} gradient.

DISCUSSION

R. prowazekii grows primarily within EC in vivo (31) with little spreading to adjacent vascular smooth muscle or fibroblasts. This study indicates that this in vivo predilection of rickettsiae for EC is probably not due to an unusual avidity of rickettsiae for EC or an increased susceptibility of EC to rickettsial infection; rickettsiae entered EC and fibroblasts similarly during a 60-min incubation. This observation is consistent with the report of Walker et al. (30) that *R. rickettsii* plaqued more efficiently on chicken embryo fibroblast monolayer cultures than they did on human umbilical cord vein-derived EC. It seems likely that rickettsiae preferentially infect EC in vivo because of the route of rickettsial introduction into the host; typhus rickettsiae (in louse feces) enter host tissue through a pore created during a louse blood meal. Once inside the microvasculature, the low temperature preference of rickettsiae (34 to 35°C) and the physical continuity of the vessel wall may restrict rickettsiae to the vascular system.

The term penetration has been widely used to describe the entry of rickettsiae into host cells. Penetration implies that rickettsiae provide the vectorial impetus for the internalization process. Indeed, some have suggested that rickettsiae actively penetrate the host cell, perhaps by use of the rickettsial phospholipase A (29, 39, 40). Whereas our data indicate that phospholipase A activity (measured as hemolytic activity) may participate in the internalization process, it does not seem likely that rickettsiae perforate the EC membrane and then enter the cell through the resultant orifice. Instead, it appears that rickettsiae enter EC as they do fibroblasts (32), via "induced phagocytosis." Unlike penetration, a term which suggests a unilateral energy requirement (rickettsial energy only), induced phagocytosis requires both rickettsial and EC activity. The first phase of this process, adherence, was at least partially dependent upon rickettsial energy; although inactivated rickettsiae adhered to EC, the adherence of killed rickettsiae to EC was reduced 50 to 80% relative to that of their viable counterparts. This requirement for rickettsial energy may have been related to the rickettsial hemolytic process; NaF, which blocked hemolysis (probably by chelating Mg^{2+} [21]), also inhibited rickettsial adherence to EC. That hemolytic activity was not an absolute requirement for adherence, however, was evident in that (i) the adherence of rickettsiae with no detectable hemolytic activity was not completely blocked, and (ii) A23187 reduced rickettsial hemolytic activity by 95% and more than doubled rickettsial adherence. There was no evidence that EC energy was required for rickettsial adherence. Rickettsial entry, on the other hand, required both rickettsial and EC energy. Treatments which blocked rickettsial hemolysis greatly inhibited rickettsial entry into EC. Concomitantly, any treatment that interfered with EC pinocytic or phagocytic activity blocked rickettsial entry. The crucial role of EC phagocytosis is especially evident in the potent inhibition of rickettsial entry by cytochalasin D; cytochalasin D has been shown to selectively block phagocytosis (11). Because rickettsial entry into EC did not occur unless rickettsiae were hemolytically active and EC were able to phagocytose, we suggest that adherent native, but not killed, rickettsiae produce an energy-dependent (rickettsial energy) signal that induces the target cell to selectively phagocytize adherent rickettsiae.

The nature of the signal for selective phagocytosis of rickettsiae is not known. It seems likely that a Ca^{2+} gradient is formed in either the rickettsiae or EC; whereas Ca^{2+} ionophore A23187 had no effect on basal EC pinocytosis, it inhibited rickettsial entry by more than 70% and stimulated rickettsial adherence to EC. This suggests that A23187 interferes with the signal for selective rickettsial entry rather than inhibiting adherence or the phagocytic mechanism per se. The observation that rickettsial hemolytic activity was also inhibited by ionophore may indicate that the rickettsial phospholipase is an active participant in production of the selective phagocytic signal. We are continuing our examination of this phenomenon.

Past investigations of rickettsia-target cell interactions focused on rickettsial interactions with such diverse target cells as erythrocytes (21–23, 38, 39, 41), lymphoblasts (6), and fibroblasts (6, 26, 32, 40, 42, 44). The development of EC

culture systems has now allowed direct examination of rickettsial interactions with their primary in vivo target cell as well as retrospective evaluation of non-endothelial model systems. Our results indicate that each non-endothelial target cell model system provided an incomplete picture of the mechanism of rickettsial entry into host cells. These non-endothelial model systems disagreed among themselves concerning the energy requirements (rickettsial and target cell) for both adherence and entry as well as concerning the specificity of rickettsial binding to the target cell. Thus, whereas the erythrocyte model correctly predicted energy requirements for rickettsial adherence to EC (23, 37), this model could not be used to examine rickettsial entry; rickettsiae do not enter erythrocytes. The more complete L-cell model, however, did not correctly predict rickettsial energy requirements during adherence to EC (32) and suggested that rickettsiae bind to a site that is insensitive to cholesterol-blocking agents (S. Walker, unpublished observation). We have not examined the specificity of rickettsial binding to EC. An additional limitation of non-endothelial target cell models is that EC differ markedly from other cell types in native activities; EC produce chemical effectors which modulate hemostasis and thrombogenesis (3, 4, 5, 9, 12, 13, 19, 20, 35).

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