Differential Requirements for Enriched Atmospheric Carbon Dioxide Content for Intracellular Growth in Cell Culture Among Selected Members of the Genus *Rickettsia*

ANNA I. KOPMANS-GARGANTIEL† AND CHARLES L. WISSEMAN, JR.*

Department of Microbiology, School of Medicine, University of Maryland at Baltimore, Baltimore, Maryland 21201

In an in vitro chicken embryo cell culture system, strains of *Rickettsia prowazekii*, *R. mooseri*, and *R. rickettsii*, but not of *R. tsutsugamushi*, required an atmosphere enriched in CO₂ for intracellular growth.

Despite the advances in recent years in methods for quantitative studies of the growth of members of the genus *Rickettsia*, still regarded as obligate intracellular parasitic bacteria, and in knowledge about independent metabolic properties of rickettsial suspensions freed from host cell components (3, 16-19), relatively little has been added to our knowledge of the nutritional requirements or of the nature of the dependency(ies) on the host cell for the various rickettsiae beyond that which was established more than two decades ago in cell cultures in Smadel's laboratory (3, 5) and in purified suspensions in Bovarnick's laboratory (e.g., 1, 2).

As a working hypothesis, it is reasonable to assume that the nutritional requirements of different members of the genus *Rickettsia* might include both substances derived essentially unaltered from the medium and substances synthesized by the host cell. One of the major problems has been that of designing experiments which would clearly distinguish, on the one hand, between the requirements of the host cell and the indirect effects these have on providing special nutrients or conditions for rickettsial growth and, on the other hand, the specific intrinsic nutritional requirements of the rickettsiae themselves. There is no reason to assume that the diverse organisms included currently in the genus *Rickettsia* have identical nutritional requirements from either medium or host cell.

An atmosphere enriched in CO₂ is a growth requirement of some bacteria, and for some of these, specific metabolic pathways for CO₂ utilization have been defined (10, 14). Among the rickettsiae pathogenic for humans, a CO₂ requirement for aerobic growth has been established only for *Rochalimaea quintana* (8), which is capable of growth in cell-free complex medium (8, 15) and has a tendency for a pericellular association with the cells of the louse gut (6) and with cells in culture (7), but without

a clearly defined capacity for facultative intracellular parasitism. The present study, which makes use of a host cell that, in culture under our conditions, does not require an extrinsic source of CO₂ for normal growth, clearly shows that the intracellular growth of some members of the genus *Rickettsia*, as currently constituted, requires an extrinsic source of CO₂ in the atmosphere, whereas that of others does not.

Selected representatives of the three major recognized groups of the genus *Rickettsia* were included in this study. In the case of the typhus and spotted fever groups, seeds were suspensions of plaque-purified organisms propagated in the yolk sac of embryonated specific-pathogen-free chicken eggs (SPAFAS, Inc., Norwich, Conn.) and included the following organisms: both the old, long-passaged attenuated E strain and a recent virulent isolate (Burundi-V-59) of *Rickettsia prowazekii*, the Wilmington strain of *Rickettsia mooseri* (*R. typhi*), and the Sheila Smith strain of *Rickettsia rickettsii*, the last two organisms being established laboratory strains. Representatives of the scrub typhus group were not plaque purified and were propagated in the yolk sac of conventional embryonated chicken eggs (Truslow Farms, Chestertown, Md.). These included the old, established laboratory Gilliam strain of *Rickettsia tsutsugamushi* and a recent field isolate from Pakistan, the Sialkot (JC-472B) strain, which was previously serotyped as Karp-like (12) but which has recently been shown to exhibit some differences in polyacrylamide polypeptide profile from the prototype Karp serotype (B. A. Hanson et al., unpublished data). Rickettsial growth studies were carried out in X-irradiated secondary chicken embryo (CE) cells infected in suspension and plated in four-chambered Lab-Tek chamber slides (Lab-Tek Products, Naperville, Ill.) to give cultures of isolated infected cells as previously described (18, 19). About 3×10^4 to 6×10^4 CE cells were plated per well, which had a surface area of approximately 2 cm². The culture medium vol-

† Present address: Saint Joseph's Hospital and Medical Center, Paterson, NJ 07503.

ume was 0.6 ml. The cell number, surface area, and volume relationships are recorded because they may influence the concentration to which CO_2 of endogenous origin may rise. The basic medium was half-strength Dulbecco modification of Eagle basic medium with Earle salts, 0.1% glucose, and 10% bovine serum. Preliminary studies (not shown) indicated that the lot of locally produced bovine serum employed supported rickettsial growth equivalent to that usually observed with commercially obtained fetal calf serum. The buffer system, however, was varied to accommodate the conditions under test. Thus, the conventional bicarbonate-carbon dioxide system was used as a standard of reference. When the CO_2 concentration was varied, bicarbonate was excluded from the medium, and pH was controlled with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), adjusted with NaOH so that the pH of the medium was maintained between 7.1 and 7.2 in the presence or absence of added CO_2 in the atmosphere. The atmosphere in the incubators was either air or air supplemented with 5% CO_2 , as indicated. The temperature of incubation was 32°C . Growth studies of unirradiated CE cells with different buffers and atmospheres were carried out in 60-mm plastic petri dishes (Falcon Plastics, Oxnard, Calif.). Approximately 10^5 CE cells was plated per dish in the conventional fashion. The buffer system and atmosphere were varied as desired. At intervals of incubation at 32°C , the cells were removed from the petri dishes with trypsin-ethylenediamine-tetraacetic acid and were counted in a hemacytometer.

Preliminary studies showed that uninfected, unirradiated CE cells, i.e., the host cells, grew as well in plastic petri dishes in medium containing HEPES buffer, with or without 5% CO_2 in the atmosphere, as they did in medium containing the conventional bicarbonate buffer system with 5% CO_2 in the atmosphere (Fig. 1), results consistent with the findings of Shipman (11), who showed that CE cells cultivated in a HEPES-containing medium in air showed no morphological changes. Likewise, a recent isolate of *R. prowazekii* (Burundi-V-59) grew equally well in irradiated CE cells in slide chamber cultures incubated in a 5% CO_2 atmosphere regardless of whether the pH was maintained by the conventional bicarbonate system or by 25 mM HEPES (Fig. 2). Thus, the HEPES buffer did not exert an inhibitory effect on the growth of either the CE host cells or a strain of *R. prowazekii*. Moreover, the CE cells did not require supplemental CO_2 in the atmosphere for growth in HEPES-buffered medium.

When intracellular rickettsial growth was examined in HEPES-buffered cultures incubated

FIG. 1. Comparison of growth of uninfected CE cells in medium with conventional bicarbonate-carbon dioxide buffer and HEPES buffer with and without 5% CO_2 in the atmosphere. pH was controlled to between 7.1 and 7.2.

FIG. 2. Comparison of the growth of a recent virulent isolate of *R. prowazekii* (Burundi-V-59) in CE cell cultures (32°C) buffered with (●) conventional bicarbonate-5% carbon dioxide system (pH 7.20) or with (○) HEPES buffer with 5% CO_2 (pH 7.15) in the atmosphere.

in air with no added CO_2 , it was found that growth was inhibited in the case of both the laboratory-adapted attenuated E strain and the recent virulent isolate of *R. prowazekii*, the Wilmington strain of *R. mooseri*, and the Sheila Smith strain of *R. rickettsii* (Fig. 3). In each

FIG. 3. Influence of presence or absence of added CO_2 on growth of rickettsiae of the typhus, spotted fever, and scrub typhus groups in CE cell cultures.

case, there was a small increase in number of rickettsiae, equivalent to about one division, in the first 24 h of incubation in the absence of a CO_2 -enriched atmosphere, after which multiplication decreased nearly to zero. In each case, when 5% CO_2 was added to the atmosphere of cultures in which rickettsial growth had become nearly stationary for 24 h or more, rickettsial replication promptly resumed and quickly attained rates similar to those of cultures which had been incubated continuously in the presence of an atmosphere containing 5% CO_2 . In sharp contrast, both the old laboratory-adapted Gilliam strain and the recent Sialkot strain isolate

of *R. tsutsugamushi* grew equally well, regardless of whether or not the atmosphere contained added CO_2 (Fig. 3).

These results clearly show that, under the conditions of culture employed, members of the typhus and spotted fever groups, but not of the scrub typhus group, of the genus *Rickettsia* require an atmosphere enriched in CO_2 for intracellular growth in CE cells. pH was rigidly controlled between 7.1 and 7.2 but did not appear to be a major factor since good rickettsial growth had been obtained in early experiments (data not shown) in which the pH of the medium in the presence of CO_2 had been maintained at

values as low as 6.6. The phenomenon did not appear to be the result of selection by prolonged passage in embryonated eggs in the laboratory because both old laboratory strains and recent isolates gave the same divergent results in the case of *R. prowazekii* and *R. tsutsugamushi*. No attempt was made to determine the minimum or optimum CO₂ concentration in the atmosphere, nor was the CO₂ requirement for replication examined in any host cell other than secondary CE cells.

Although not actually measured in the case of the CE cells under the conditions of culture employed in these studies, it is reasonable to assume that CO₂ was a by-product of host cell metabolism. In any case, neither the CO₂ produced by isolated CE cells in our cultures nor the CO₂ naturally present in air (~0.2 to 0.3%) was sufficient to supply the needs for intracellular replication of the members of the typhus and spotted fever groups of the genus *Rickettsia* that were tested in this study. It is unknown whether the endogenous CO₂ produced by higher densities of cells, approaching conditions which obtain in solid tissues or in blood in capillaries and postcapillary venules, would be sufficient to supply the needs of these organisms. Since extrinsic CO₂ has been shown to enter metabolic pathways in some vertebrate cells (9), the present studies cannot conclusively distinguish between an intrinsic, direct requirement by these rickettsiae for supplemental CO₂ and an indirect effect through the host cell.

The fact that all members of the typhus and spotted fever groups that were tested undergo a limited amount of replication, equivalent approximately to one division, upon transfer to the CO₂-deficient environment is intriguing. The possibility cannot be excluded at this time that this simply represents depletion of the CO₂ originally present in the system. On the other hand, it is also consistent with a specific CO₂-dependent stage in the growth cycle. Thus, organisms having passed this stage in the growth cycle may continue normal replication until the CO₂-dependent stage is again encountered. This may have practical potential for synchronizing rickettsial cultures.

It is unknown whether strains of *R. tsutsugamushi* are devoid of a requirement for supplemental CO₂ or if the endogenous CO₂ of host cell origin plus the CO₂ in air is sufficient to satisfy their needs. Regardless of whether the differences in extrinsic CO₂ requirement are qualitative or quantitative, it is clear that the scrub typhus group differs strikingly from the typhus and spotted fever groups in this property, as it does in certain other properties (4, 13).

We gratefully acknowledge the superior technical assist-

ance and advice of A. Waddell, M. L. Cremer, M. Jones, and M. Doelp and excellent manuscript typing by C. Queen.

This study received financial support from contract DADA 17-71-C-1007 with the U.S. Army Medical Research and Development Command. A. I. Kopmans-Gargantiel was a predoctoral trainee supported by Public Health Service training grant 5 TO1 AI00016 from the National Institutes of Health.

LITERATURE CITED

1. Bovarnick, M. R. 1960. Incorporation of acetate-1-C¹⁴ into lipid by typhus rickettsiae. *J. Bacteriol.* 80:508-512.
2. Bovarnick, M. R., and L. Schneider. 1960. The incorporation of glycine-1-C¹⁴ by typhus rickettsiae. *J. Biol. Chem.* 235:1727-1731.
3. Bozeman, F. M., H. E. Hopps, J. X. Danauskas, E. B. Jackson, and J. E. Smadel. 1956. Study on the growth of rickettsiae. I. A tissue culture system for quantitative estimations of *Rickettsia tsutsugamushi*. *J. Immunol.* 76:475-488.
4. Gimenez, D. F. 1961. Staining rickettsiae in yolk sac cultures. *Stain Technol.* 39:135-140.
5. Hopps, H. E., E. B. Jackson, J. X. Danauskas, and J. E. Smadel. 1959. Study on the growth of rickettsiae. III. Influence of extracellular environment on the growth of *Rickettsia tsutsugamushi* in tissue culture cells. *J. Immunol.* 82:161-171.
6. Ito, S., and J. W. Vinson. 1965. Fine structure of *Rickettsia quintana* cultivated in vitro and in the louse. *J. Bacteriol.* 89:481-495.
7. Merrell, B. R., E. Weiss, and G. A. Dasch. 1978. Morphological and cell association characteristics of *Rochalimaea quintana*: comparison of the Vole and Fuller strains. *J. Bacteriol.* 135:633-640.
8. Myers, W. F., L. D. Cutler, and C. L. Wisseman, Jr. 1969. Role of erythrocytes and serum in the nutrition of *Rickettsia quintana*. *J. Bacteriol.* 97:663-666.
9. Raghunatan, R., J. D. Russell, and I. J. Arinze. 1977. Pyruvate carboxylase and phosphoenolpyruvate carboxykinase in cultured human fibroblasts. *J. Cell. Physiol.* 92:283-292.
10. Repaske, R., and M. A. Clayton. 1978. Control of *Escherichia coli* growth by CO₂. *J. Bacteriol.* 135:1162-1164.
11. Shipman, C., Jr. 1969. Evaluation of 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) as a tissue culture buffer. *Proc. Soc. Exp. Biol. Med.* 130:305-310.
12. Shirai, A., and C. L. Wisseman, Jr. 1975. Serologic classification of scrub typhus isolates from Pakistan. *Am. J. Trop. Med. Hyg.* 24:145-153.
13. Silverman, D. J., and C. L. Wisseman, Jr. 1978. Comparative ultrastructural study on the cell envelopes of *Rickettsia prowazekii*, *Rickettsia rickettsii*, and *Rickettsia tsutsugamushi*. *Infect. Immun.* 21:1020-1023.
14. Stanier, R. Y., M. Doudoroff, and E. A. Adelberg. 1970. *The microbial world*, 3rd ed. Prentice-Hall, Englewood Cliffs, N.J.
15. Vinson, J. W. 1966. *In vitro* cultivation of the rickettsial agent of trench fever. *Bull. W.H.O.* 35:155-164.
16. Weiss, E. 1973. Growth and physiology of rickettsiae. *Bacteriol. Rev.* 37:259-283.
17. Weiss, E., L. W. Newman, R. Grays, and A. E. Green. 1972. Metabolism of *Rickettsia typhi* and *Rickettsia akari* in irradiated L cells. *Infect. Immun.* 6:50-57.
18. Wisseman, C. L., Jr., E. A. Edlinger, A. D. Waddell, and M. R. Jones. 1976. Infection cycle of *Rickettsia rickettsii* in chicken embryo and L-929 cells in culture. *Infect. Immun.* 14:1052-1064.
19. Wisseman, C. L., Jr., and A. D. Waddell. 1975. *In vitro* studies on rickettsia-host cell interactions: intracellular growth cycle of virulent and attenuated *Rickettsia prowazekii* in chicken embryo cells in slide chamber cultures. *Infect. Immun.* 11:1391-1401.