INFECTION AND IMMUNITY, May 1996, p. 1858–1861 Vol. 64, No. 5
0019-9567/96/$04.00+0

Uptake and Killing of Lyme Disease and Relapsing Fever Borreliae in the Perfused Rat Liver and by Isolated Kupffer Cells

VITTORIO SAMBRI,[1] RITA ALDINI,[2] FRANCESCA MASSARIA,[1] MARCO MONTAGNANI,[3] SILVIA CASANOVA,[4] AND ROBERTO CEVENINI[1]*

Istituto di Microbiologia,[1] Istituto di Scienze Chimiche,[2] and Cattedra di Gastroenterologia,[3] University of Bologna, and Laboratorio di Microscopia Elettronica, Ospedale M. Malpighi,[4] Bologna, Italy

Received 20 September 1995/Returned for modification 1 November 1995/Accepted 7 February 1996

In situ-perfused rat livers were infused with a single dose of 1.5×10^7 radiolabeled borreliae. Significant ($P < 0.00005$) differences in the liver uptake of the agents of Lyme borreliosis, *Borrelia burgdorferi* IRS, *Borrelia afzelii* VS461, and *Borrelia garinii* PBi, and that of the agents of relapsing fever, *Borrelia hermsii*, *Borrelia parkeri*, and *Borrelia turicatae*, were observed. The liver uptakes ranged between 65.9% for *B. burgdorferi* IRS and 40.5% for *B. turicatae*. Neither relapsing fever nor Lyme disease borreliae were recovered from infected livers when the livers were cultured in Barbour-Stoenner-Kelly II medium. The in vitro uptake of *B. burgdorferi* IRS by isolated rat Kupffer cells was rapid, and within 30 min of the infection, large intracellular aggregates of amorphous material were detectable by immunofluorescence with specific anti-*B. burgdorferi* antibody. The reculturing of *B. burgdorferi* IRS from Kupffer cells incubated for 24 h in RPMI medium before inoculation with bacteria was negative. The results obtained in this study indicated that borreliae are efficiently taken up and killed by rat hepatic macrophages in the absence of serum factors.

The diagnosis of most borrelioses is primarily based on the detection of spirochetes in the peripheral blood of febrile persons or animals, which suggests that there are spirochetemias that are not well defined so far as time course and concentration are concerned. Spirochetemias due to *Borrelia burgdorferi* are mainly detectable in the early stages of Lyme disease; however, they may also occur later in the disease (23). Relapsing fever borreliae are detectable in the blood of patients during pyrexia (8). In vitro studies of spirochetes show that their interaction with phagocytes is quite as expected (5, 15) and similar to that for many other microorganisms. Although the importance of the hepatic reticuloendothelial system in host defense by the phagocytosis and killing of blood-borne pathogens has been well established, so far as we know, there are no data about borreliae.

The perfused liver has been used several times in the past decades to study bacterial hepatic phagocytosis (6, 14, 17). Therefore, we used such a technique to evaluate two specific aspects of the hepatic reticuloendothelial system in the elimination of circulating bacteria: uptake and killing. In addition, the abilities of isolated Kupffer cells (KC) to associate with *B. burgdorferi* in vitro were also evaluated.

Although the mouse is the most widely used model for experimental borrelioses, this study was performed with rats, since they are more suitable for liver perfusion and cell separation because of their larger liver size. The applicability of rats for experimental studies of *Borrelia* infection has also been shown (4, 8, 12).

Bacterial strains, culture conditions, and labeling. The following *Borrelia* strains were used: *Borrelia hermsii* HS-1 (ATCC 35209), *Borrelia parkeri* M3001, *Borrelia turicatae*, *B. burgdorferi* IRS (ATCC 35211), *Borrelia garinii* PBi, and *Borrelia afzelii* VS461. Borreliae were cultured in Barbour-Stoenner-Kelly (BSK) II medium at 34°C as previously reported (21). When required, spirochetes were grown in the presence of 1 μCi of ^{14}C-labeled amino acid mixture (>50 mCi/ml; Amersham Co., Amersham, United Kingdom) per ml for 96 h, washed three times with Krebs-Ringer solution (see below), counted in a Petroff-Hausser chamber, resuspended in Krebs-Ringer solution at a concentration of 1.5×10^6 motile organisms per ml, and used within 1 h to perfuse rat livers.

Experimental animals. Male Sprague-Dawley rats (body weight, 180 to 220 g) were used as liver donors. Food was withdrawn the evening before the experiment; water was available ad libitum.

Liver perfusion. The technique of rat liver perfusion has already been described (1) and was performed by following the method of Mortimore (18). Briefly, the animals were anesthetized intraperitoneally with pentobarbital sodium (50 mg/kg of body weight), and the livers were perfused through the portal vein, with the effluent being collected from the inferior vena cava immediately above the sovrahepatic veins. The perfusate was Krebs-Ringer bicarbonate solution containing glucose (5.55 mmol/liter) with bovine serum albumin (3%, wt/vol) (fraction V; Sigma Chemical Co., St. Louis, Mo.). The complete blanching of all liver lobes indicated satisfactory perfusion. Oxygenation was done with O_2 plus CO_2 (95/5, vol/vol), with Silastic tubing being used (Dow-Corning, Midland, Mich.). The temperature and pH of the perfusate leaving the liver were monitored. The perfusate flow was established as 2.2 to 2.9 ml min^{-1} g liver^{-1}. The portal vein pressure was constant at 12 cm of water, no significant change in the aspartate aminotransferase levels (12.0 ± 2.0 IU/liter) was observed throughout the experiment, and the pH of the effluent from the liver ranged between 7.36 and 7.42. During each perfusion, one dose of 1.5×10^7 radiolabeled (150,000 ± 30,000 cpm) bacteria in 10 ml of Krebs-Ringer solution was infused into the portal vein. The effluent from the livers was collected at 1-min intervals over 30 min in preweighted tubes immediately after the infusion. At the end of each experiment, radioactivity was

* Corresponding author. Mailing address: Istituto di Microbiologia, Ospedale S. Orsola, Via Massarenti 9, 40138 Bologna, Italy. Phone: 39/51/341652. Fax: 39/51/341632.

measured by directly applying 100 μl of each outflow sample to 0.45-μm-pore-size cellulose acetate membrane filters and counting the radioactivity with a Packard 2000 CA liquid scintillation analyzer after the addition of Ready-Solv EP (Beckman). The cumulative radioactivity throughout the experiment represented the amount of bacteria escaping the liver uptake. A 30-min period was chosen in order to rule out bacterial efflux from the liver at later times.

Expression of the results of liver uptake of borreliae. The hepatic uptake was expressed as a percentage of the administered radioactivity and was calculated as 100% minus the percentage of outflow. The statistical analysis was performed by the Wilcoxon test for independent data.

Quantitation of the hepatic concentration of viable borreliae. By a sterile technique, the liver was excised from the rat and homogenized in 10 ml of BSK II medium. One milliliter of homogenate was diluted in 10 ml of BSK II medium, and serial 10-fold dilutions in BSK II medium were done. The cultures were incubated at 34°C for 3 weeks.

Separation of KC. KC were harvested as previously reported by Crocker et al. (11), with minor modifications. Briefly, rat livers were first perfused with 50 ml of calcium- and magnesium-free Hanks balanced salt solution which had been prewarmed to 37°C and buffered at pH 7.3 with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (Sigma Chemicals). The livers were then perfused with 50 ml of complete Hanks balanced salt solution containing 0.05% collagenase (type IV; Sigma) that had been prewarmed to 37°C. The livers were then excised, chopped finely with scissors, and suspended in 20 ml of the collagenase solution described above supplemented with 100 μg of DNase (type I; Sigma) per ml. After digestion for 10 min at 37°C, the cell suspension was passed through a fine sieve. The cells were washed three times with Ca- and Mg-free Hanks balanced salt solution by centrifugation at 250 × *g* for 10 min, suspended in 2 ml of buffer, and layered over 5 ml of isotonic Percoll solution with a density of 1.037 g/ml. After centrifugation at 650 × *g* for 30 min, the pelleted cells were washed and finally resuspended in culture medium (RPMI 1640 with 20% fetal calf serum) at a concentration of 3.0×10^6 cells per ml. A 0.5-ml portion of the cell suspension was added to glass coverslips (13-mm diameter). Macrophages were selected by allowing them to adhere for 2 h at 37°C under 5% CO_2. After nonadherent cells were removed by gentle washing, adherent cells were incubated with RPMI 1640 for 24 h before uptake and killing experiments were performed. More than 95% of the adherent cells were esterase positive.

Uptake and killing of borreliae by isolated KC. The uptake of borreliae by KC was studied by indirect immunofluorescence assay as follows. *B. burgdorferi* IRS cells (2×10^7 cells per coverslip) were incubated with adherent macrophages in RPMI 1640 for 5, 15, 30, or 60 min at 37°C, washed in phosphate-buffered saline (pH 7.4), fixed in cold acetone, and incubated for 30 min with mouse immune ascitic fluid anti-*B. burgdorferi* (10). Bacterial cells were visualized with anti-mouse fluorescein-conjugated antibody (Dako, Copenhagen, Denmark). For the recovery of macrophage-associated spirochetes, *B. burgdorferi* cells (2×10^7 cells per coverslip) were added to adherent macrophages and incubated for 2 h at 37°C. Incubations were terminated by thoroughly rinsing infected monolayers with RPMI 1640 for the removal of free spirochetes. The cells were then scraped off and assayed for bacterial viability by culturing them in BSK II medium at 34°C for 3 weeks.

Electron microscopy. Liver sections were fixed for transmission electron microscopy according to standard procedures. Twenty milliliters of 2.5% glutaraldehyde in 0.1 M sodium cacodylate was infused directly into the portal vein. Approximately 25 1-mm^3 sections of the liver were placed in the same fixative overnight. After fixation, the liver slices were rinsed in cacodylate buffer (pH 7.4), postfixed with osmium tetroxide, embedded in Epon, and sliced. Thin sections were stained with uranyl acetate and lead citrate and observed with an electron microscope (JEOL 100CX).

The motility and viability of *Borrelia* strains in Krebs-Ringer solution were preliminarily evaluated. Bacteria suspended in Krebs-Ringer solution for 1, 2, 4, and 8 h were studied by dark-field microscopy and by culture. No reduction in the number of motile organisms or viable bacteria in comparison with controls suspended in BSK II medium was observed.

The results obtained after a single infusion of 1.5×10^7 radiolabeled bacteria through the perfused rat liver, which had previously been washed free of blood, are reported in Fig. 1. The liver uptake, expressed as the percentage of radioactivity retained by the liver, ranged from 65.9% for *B. burgdorferi* IRS to 40.5% for *B. turicatae*. The remaining *Borrelia* species gave intermediate values. The differences in liver uptake for the agents of Lyme disease (*B. burgorferi* IRS, *B. garinii* PBi, and *B. afzelii* VS461) and the agents of relapsing fever (*B. turicatae*, *B. parkeri*, and *B. hermsii*) were statistically significant ($P < 0.00005$), suggesting that the different compositions and structures of the bacterial surfaces (2, 3, 7, 9, 19, 20) are likely to play a crucial role in the bacterium-phagocyte interaction. In fact, relapsing fever borreliae escaped hepatic uptake by the liver reticuloendothelial system to a significantly higher extent than did the agents of Lyme disease. This observation may be of some relevance, considering that in vivo bacteremias are more frequently detectable in patients infected with relapsing fever borreliae than in patients with *B. burgdorferi* infections.

The kinetics of the radioactivity appearing in the liver effluents after portal infusion of Lyme borreliosis and relapsing fever agents are given in Fig. 2. Borreliae appeared in the effluent immediately after the infusion, and their numbers increased to a maximum value within the second minute of injection, after which they decreased to a baseline almost approaching zero. No diffusion of bacteria back into the perfusate was detected, at least throughout 30 min. Under the present experimental conditions, the hepatic uptake was far from saturation, since an increase of the borrelia load to the liver by 100-fold (2.5×10^9) reduced the hepatic uptake by only 10-fold (data not reported).

Pretreatment of *B. burgdorferi* IRS with specific antibody (indirect immunofluorescence assay titer, 1:4,096) obtained from an immunized rat increased the liver uptake by 21.5%, whereas heat inactivation (56°C for 1 h) did not modify the uptake compared with that with untreated bacteria. The uptake was also unchanged when livers from previously vaccinated rats (four intramuscular injections of 35 μg of *B. burgdorferi* IRS proteins) were perfused with viable spirochetes.

In order to evaluate the recovery of infused bacteria, the livers were washed, homogenized, and cultured in BSK II medium. Neither relapsing fever nor Lyme disease borreliae were recovered, showing an efficient uptake and killing of bacteria. Reculturing experiments with *Borrelia* strains mixed with homogenized livers from uninfected animals gave negative results, ruling out any possible bacteriostatic or bacteriocidal effect of materials released from disrupted liver tissue.

Electron microscopy of rat livers perfused with 1.5×10^8 *B. burgdorferi* IRS cells and fixed 30 min after the perfusion revealed the presence of bacteria in the KC but not in the sinusoids (Fig. 3), suggesting an almost immediate phagocytosis of infused bacteria by the cells of the hepatic reticuloendothelial system. Sections from livers perfused with 1.5×10^7

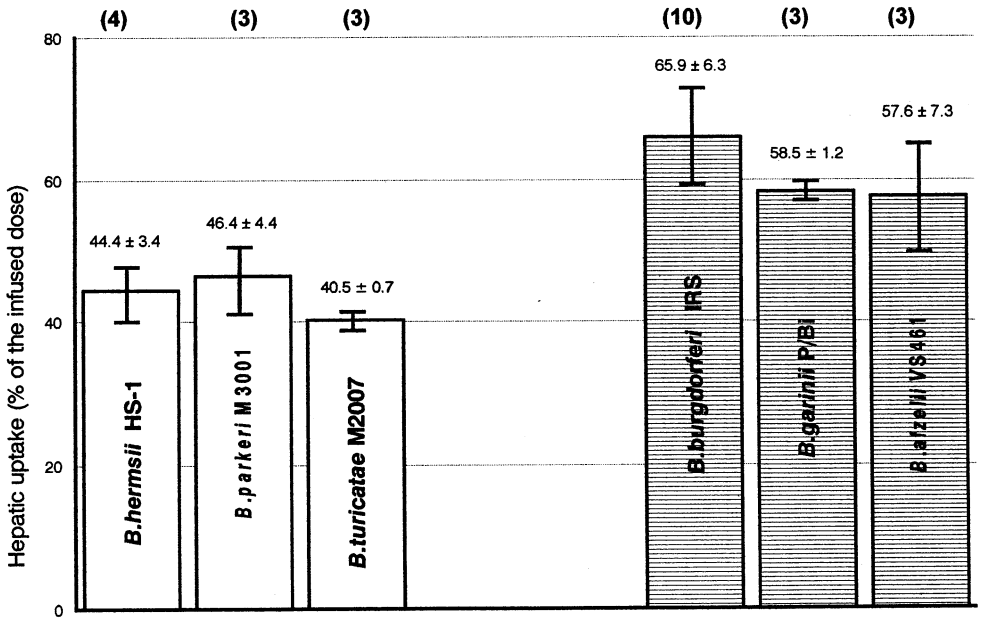

FIG. 1. Uptake of radiolabeled borreliae (percentage of the infused dose) by the liver. A single dose of 1.5×10^7 ^{14}C-labeled bacteria (150,000 ± 30,000 cpm) was infused into the portal vein, and the effluent from the sovrahepatic veins was counted for radioactivity. The data shown are the means ± standard deviations. The numbers of experiments are shown at the top in parentheses. The differences in liver uptake for the agents of Lyme disease (hatched bars) and the agents of relapsing fever (open bars) were statistically significant ($P < 0.00005$).

spirochetes were not adequate for an electron microscopy study because of the paucity of bacteria present in most of the sections.

The in vitro uptake and killing of *B. burgdorferi* IRS by KC were also studied by immunofluorescence staining of macrophage-associated bacteria and by culture of infected cells. Ingestion of spirochetes occurred rapidly (5 to 15 min within infection) and did not require opsonization (5). Within a very short time (5 min) of the addition of borreliae, the spirochetes were adherent to the KC, sometimes being elongated or wound around a cell (Fig. 4a). At a later stage (15 min), intracellular, morphologically distinct spirochetes were rarely observed, with small round fluorescent inclusion-like bodies being evident in the cells (Fig. 4b). Within 1 h, large aggregates of fluorescent material were present (Fig. 4c and d). This result confirms the results of the time course study of mouse macrophage infection with *B. burgdorferi* obtained by Montgomery et al. (16) and extends the observation to rat KC. An effective killing of phagocytosed borreliae was suggested by the impossibility of reculturing bacteria from isolated and infected KC and from livers after perfusion with *B. burgdorferi* IRS. These results, however, are not in agreement with those of Montgomery et al. (16), who documented the persistence, although only occasionally, of *B. burgdorferi* within mouse macrophages by reculturing spirochetes from macrophages after infection. The different results may be, at least in part, explained by the use of cells

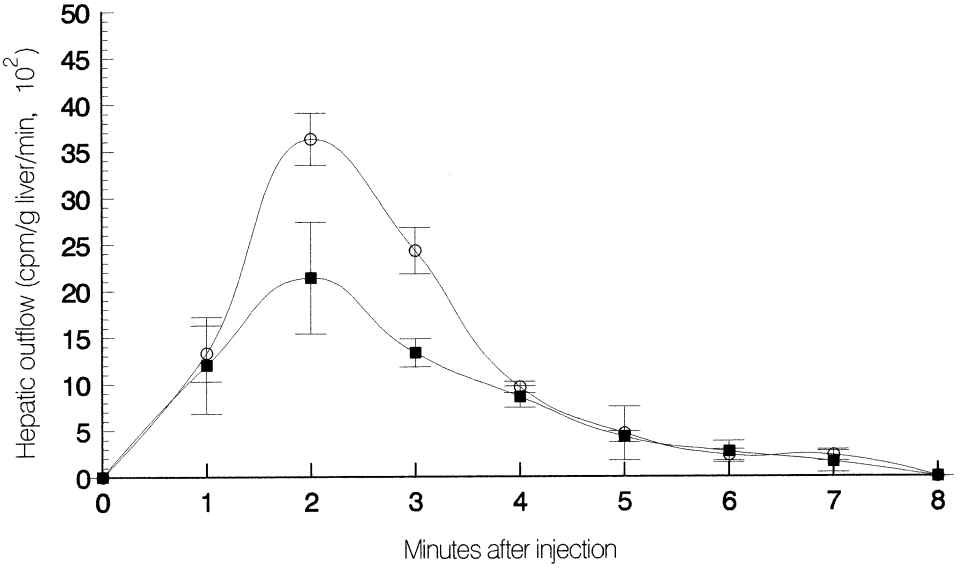

FIG. 2. Time course of the kinetic display of the radioactivity (means ± standard deviations) appearing in the effluent after infusion of 1.5×10^7 labeled bacteria (150,000 ± 30,000 cpm). Symbols: ■, Lyme borreliosis agents (*B. burgdorferi*, *B. afzelii*, and *B. garinii*); ○, relapsing fever agents (*B. hermsii*, *B. parkeri*, and *B. turicatae*).

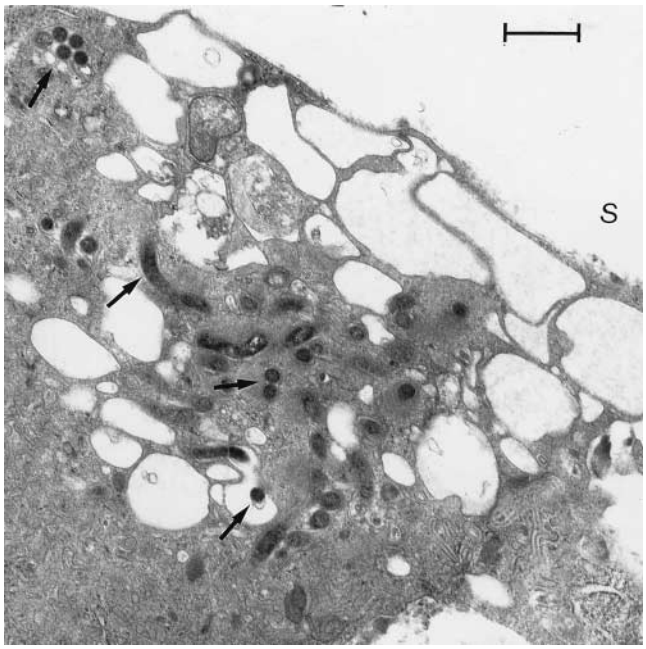

FIG. 3. Transmission electron micrograph showing phagocytosis of *B. burgdorferi* IRS by a KC. The fixative was directly perfused through the portal vein 30 min after the infusion of 1.5×10^8 spirochetes into the rat liver. Many intracellular borreliae (some singled out by arrows) are present. The liver sinusoid (S) is free of bacteria. Bar, 1 μm.

from either different animals or murine macrophage cell line J774 (16) instead of freshly prepared macrophages. In fact, an established macrophage cell line may have either modified or lost some of the phagocytic abilities expressed by the original cells.

The results obtained in both the in vivo and in vitro studies of the borrelial uptake and killing capacity of rat KC indicate that spirochetes are rapidly taken up and killed by rat hepatic macrophages in the absence of serum factors, demonstrating that borreliae are highly susceptible to nonopsonic phagocytosis in vivo as well as in vitro by rat liver macrophages. Nonopsonic phagocytosis may play an important role in host defense against infection, especially in the initial phase of infection, at sites where levels of antibody and complement are naturally low. Nonspecific clearance is also important in host defense against bacteremia (13, 22), with such clearance being a prominent feature of the natural history of *Borrelia* infections.

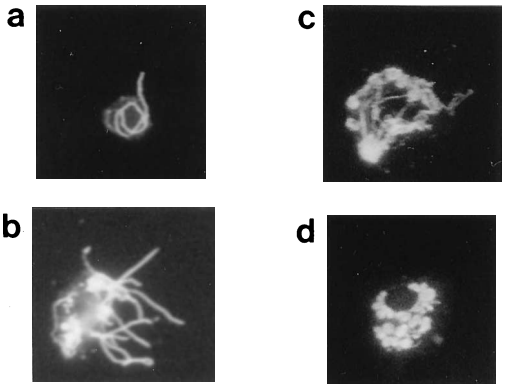

FIG. 4. Photomicrograph of time course infection of isolated rat KC by *B. burgdorferi* IRS stained by immunofluorescence with specific mouse antibodies. (a) A spirochete wound around a rat KC 5 min after infection. (b) Spirochetes adherent to a KC and spirochete-derived inclusion-like bodies inside the cell 15 min after infection. (c and d) Spirochete-derived inclusion-like bodies 30 (c) and 60 (d) min after infection.

Editor: R. E. McCallum

REFERENCES

1. **Aldini, R., A. Roda, P. Simoni, P. Lenzi, and E. Roda.** 1988. Uptake of bile acids by perfused rat liver: evidence of a structure-activity relationship. Hepatology **10:**840–845.
2. **Barbour, A. G., S. L. Tessier, and S. F. Hayes.** 1984. Variation in a major surface protein of Lyme disease spirochetes. Infect. Immun. **45:**94–100.
3. **Barstad, P. A., J. E. Coligan, M. G. Raum, and A. G. Barbour.** 1985. Variable major proteins of *Borrelia hermsii*: epitope mapping and partial sequence analysis of CNBr peptides. J. Exp. Med. **161:**1302–1311.
4. **Barthold, S. W., K. D. Moody, J. A. Terwilliger, P. H. Duray, R. O. Jacoby, and A. C. Steere.** 1988. Experimental Lyme arthritis of rats infected with *Borrelia burgdorferi*. J. Infect. Dis. **157:**842–846.
5. **Benach, J. L., H. B. Fleit, G. S. Habicht, J. L. Coleman, E. M. Bosler, and B. P. Lane.** 1984. Interactions of phagocytes with the Lyme disease spirochete: role of the Fc receptor. J. Infect. Dis. **150:**497–507.
6. **Bonventre, P. F., and E. Oxman.** 1965. Phagocytosis and intracellular disposition of viable bacteria by the isolated perfused rat liver. J. Reticuloendothel. Soc. **2:**313–325.
7. **Brandt, M. E., B. S. Riley, J. D. Radolf, and M. V. Norgard.** 1990. Immunogenic integral membrane proteins of *Borrelia burgdorferi* are lipoproteins. Infect. Immun. **58:**983–991.
8. **Burgdorfer, W.** 1976. The diagnosis of the relapsing fevers, p. 225–234. *In* R. C. Johnson (ed.), The biology of parasitic spirochetes. Academic Press, New York.
9. **Carter, C. J., S. Bergström, S. J. Norris, and A. G. Barbour.** 1994. A family of surface-exposed proteins of 20 kilodaltons in the genus *Borrelia*. Infect. Immun. **62:**2792–2799.
10. **Cevenini, R., V. Sambri, F. Massaria, R. Franchini, A. D'Antuono, G. Borda, and M. Negosanti.** 1992. Surface immunofluorescence assay for diagnosis of Lyme disease. J. Clin. Microbiol. **30:**2456–2461.
11. **Crocker, P. R., J. M. Blackwell, and D. J. Bradley.** 1984. Expression of the natural resistance gene *Lsh* in resident liver macrophages. Infect. Immun. **43:**1033–1040.
12. **Galbe, J. L., E. Guy, J. M. Zapatero, E. I. B. Peerschke, and J. L. Benach.** 1993. Vascular clearance of *B. burgdorferi* in rats. Microb. Pathog. **14:**187–202.
13. **Hirakata, Y., K. Tomono, K. Tateda, T. Matsumoto, N. Furuya, K. Shimoguchi, M. Kaku, and K. Yamaguchi.** 1991. Role of bacterial association with Kupffer cells in occurrence of endogenous systemic bacteremia. Infect. Immun. **59:**289–294.
14. **Klein, A., M. Zhadkewich, J. Margolick, J. Winkelstein, and G. Bulkley.** 1994. Quantitative discrimination of hepatic reticuloendothelial clearance and phagocytic killing. J. Leukocyte Biol. **55:**248–252.
15. **Montgomery, R. R., and S. E. Malawista.** 1994. Degradation of *Borrelia burgdorferi* within macrophage lysosomes: an ultrastructural study, p. 84–87. *In* R. Cevenini, V. Sambri, and M. La Placa (ed.), Advances in Lyme borreliosis research. Esculapio, Bologna, Italy.
16. **Montgomery, R. R., M. H. Nathanson, and S. E. Malawista.** 1993. The fate of *Borrelia burgdorferi*, the agent for Lyme disease, in mouse macrophages. J. Immunol. **150:**909–915.
17. **Moon, R. J., R. A. Vrable, and J. A. Broka.** 1975. In situ separation of bacterial trapping and killing functions of the perfused liver. Infect. Immun. **12:**411–418.
18. **Mortimore, G. E.** 1961. Effect of insulin on potassium transfer in isolated rat liver. Am. J. Physiol. **200:**1315–1319.
19. **Sambri, V., and R. Cevenini.** 1992. Incorporation of cysteine by *Borrelia burgdorferi* and *Borrelia hermsii*. Can. J. Microbiol. **38:**1016–1021.
20. **Sambri, V., A. Marangoni, F. Massaria, A. Farencena, M. La Placa, Jr., and R. Cevenini.** 1995. Functional activities of antibodies directed against surface lipoproteins of *Borrelia hermsii*. Microbiol. Immunol. **39:**623–627.
21. **Sambri, V., C. Stefanelli, C. Rossoni, M. La Placa, Jr., and R. Cevenini.** 1993. Acylated proteins in *Borrelia hermsii*, *Borrelia parkeri*, *Borrelia anserina*, and *Borrelia coriaceae*. Appl. Environ. Microbiol. **59:**3938–3940.
22. **Speert, D. P., B. A. Loh, D. A. Cabral, and I. E. Salit.** 1986. Nonopsonic phagocytosis of nonmucoid *Pseudomonas aeruginosa* by human neutrophils and monocyte-derived macrophages is correlated with bacterial piliation and hydrophobicity. Infect. Immun. **53:**207–212.
23. **Wilske, B., and V. Preac-Mursic.** 1993. Microbial diagnosis of Lyme borreliosis, p. 267–299. *In* K. Weber and W. Burgdorfer (ed.), Aspects of Lyme borreliosis. Springer-Verlag, Berlin.