ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1982, p. 823–829
0066-4804/82/050823-07$02.00/0

Vol. 21, No. 5

Action of Penicillin on *Borrelia hermsii*

ALAN G. BARBOUR,* WILLIAM J. TODD, AND HERBERT G. STOENNER

National Institute of Allergy and Infectious Diseases, Laboratory of Microbial Structure and Function, Rocky Mountain Laboratories, Hamilton, Montana 59840

Received 25 November 1981/Accepted 29 January 1982

Borrelia hermsii, a spirochete and an etiological agent of relapsing fever, was cultivated in modified Kelly medium. Studies of the action of penicillin on *B. hermsii* strain HS1 revealed the following: (i) the in vitro minimum inhibitory concentration and minimum bactericidal concentration of benzylpencillin for this strain were 0.4 and 3.1 nmol/ml (0.15 and 1.1 μg/ml), respectively; (ii) the primary morphological responses at the minimum bactericidal concentration of benzylpenicillin were the formation of spheroplast-like structures and an increased number of small, membranous blebs; (iii) radioactive benzylpenicillin bound to five penicillin-binding proteins in the whole cells of *B. hermsii*. The 50% binding concentrations of labeled penicillin for the five penicillin-binding proteins were within a factor of five of the minimum inhibitory concentration. More than one-half of the total bound labeled penicillin was associated with penicillin-binding protein 1, the penicillin-binding protein with the largest apparent molecular weight (90,000).

Pathogenic borreliae, the etiological agents of relapsing fever, are susceptible to penicillin in vivo. This has been observed during treatment of infections of both humans and laboratory animals (3, 9, 22, 24). *Borrelia* species thus resemble other spirochetes, including *Treponema pallidum*, in their susceptibility to penicillin (1, 2, 5, 10). However, little is known about the mechanisms of action of penicillin on spirochetes. This lack of knowledge has been due, in part, to past difficulties in the cultivation of many of these organisms. The ability to grow pathogenic strains of *Borrelia* in vitro permits examination of the effects of penicillin on this representative of spirochetes. We have specifically studied *Borrelia hermsii*, a cause of relapsing fever in North America. We have established in vitro inhibitory and bactericidal concentrations of penicillin for this *Borrelia* strain, demonstrated the morphological effect of penicillin at its minimum bactericidal concentration, and identified the penicillin-binding proteins (PBPs) in cells of this species.

MATERIALS AND METHODS

Conditions of growth. The origin of the strain of *B. hermsii* used in these studies has been described (26, 27). It was designated strain HS1. The organisms were kept at −70°C in media containing 10% glycerol. They were passaged once in mice before in vitro cultivation. Glass tubes (Pyrex no. 9826) were filled with Kelly medium (18) containing 5% (vol/vol) CMRL medium 1066 (GIBCO Laboratories, Grand Island, N.Y., no. 330-1540) and 0.2% Yeastolate (Difco Laboratories, Detroit, Mich.) (H. G. Stoenner, submitted for publication). After inoculation, the tubes were tightly capped and incubated at 35°C. Under these conditions and from an initital inoculum of 10^5 organisms per ml, this strain achieved a density of 10^7 organisms per ml after 48 h. Spirochetes were enumerated by dark-field microscopy (26).

Antibiotics. The ethylpiperidium salt of [^{3}H]benzylpenicillin ([^{3}H]penicillin), with a specific activity of 31 Ci/mmol, was the generous gift of E. Stapley and P. Cassidy, Merck & Co., Inc., Rahway, N.J. The potassium salt of benzylpenicillin was obtained from Eli Lilly & Co., Indianapolis, Ind. One nanomole of benzylpenicillin is equal to 0.37 μg.

Antibiotic susceptibility. Tubes of media containing twofold dilutions of benzylpenicillin were inoculated with exponentially growing cells to a final cell density of 10^5 per ml. After incubation for 48 h, motile spirochetes in the tubes were counted by dark-field microscopy. The minimum inhibitory concentration was the lowest concentration at which no growth occurred, i.e., at which the spirochete count was 10^5 per ml or less. To estimate the bactericidal concentration of benzylpenicillin, we inoculated three 18-day-old Swiss mice of the Rocky Mountain Laboratories stock intraperitoneally with 0.1 ml of the antibiotic-treated cultures at the time of enumeration of the spirochetes. Preliminary studies showed that as few as one to two organisms of *B. hermsii* inoculated intraperitoneally resulted in spirochetemia in this strain of mice. The mice were bled from the tail vein on days 2, 4, 6, and 8 after inoculation. The blood was examined by dark-field microscopy for the presence of motile spirochetes. Twenty or more 125× fields were examined in search of organisms. Mice inoculated with ten or fewer borreliae and followed in this way had detectable spirochetemia by day 4 to 5. The minimum bactericidal concentration was the lowest concentration which prevented spirochetemia in all three mice.

TABLE 1. Inhibitory and bactericidal concentrations of benzylpenicillin for *B. hermsii* strain HS1 in modified Kelly medium

Benzylpenicillin concn (nmol/ml)	No. of motile spirochetes per ml after 48 h	Spirochetemia in mice injected with 48-h culture
0	10^7	+
0.1	10^7	+
0.2	10^6	+
0.4	10^5	+
0.8	$<10^4$	+
1.6	$<10^4$	+
3.1	$<10^4$	−

Electron microscopy. Cultures in log-phase growth containing 10^6 cells per ml were treated with benzylpenicillin. A control culture contained no antibiotic. After 10 h of incubation, 8 ml of each culture was centrifuged at 8,000 × *g* for 20 min at 4°C. The pellets were immediately fixed for 30 min in 50 mM Na cacodylate buffer (pH 7.4) containing 2 mM CaC12, 4% paraformaldehyde, 2% gluteraldehyde, and 0.02% trinitrocresole, without previous resuspension. After this primary fixation, the samples were rinsed in the cacodylate buffer and postfixed for 30 min in the same buffer containing 2% osmium tetroxide. The fixed samples were dehydrated in a series of graded concentrations of ethanol and embedded in Spurrs embedding medium. Thin sections were stained with uranyl acetate and examined in a Hitachi HU-11 electron microscope.

Binding of labeled pencillin to isolated membranes and to whole cells. *B. hermsii* grown under the conditions described above were harvested at a cell density of 1 × 10^7 to 2 ×10^7 cells per ml. After 200 ml of pooled culture medium was centrifuged (8,000 × *g* for 20 min at 4°C), the cells in the pellet were suspended in 50 ml of cold phosphate-buffered saline (pH 7.4) and recentrifuged. The pellet was resuspended in phosphate-buffered saline to a density of 10^9 cells per ml. Cell membranes were isolated and then treated with sodium lauroyl sarcosinate (Sarkosyl) by a modification of the method of Spratt (25). Cells were disrupted by sonication in the presence of 0.5% 2-mercaptoethanol with six 15-s pulses with a Sonicator Cell Disrupter (Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) Centrifugation at 8,000 × *g* for 20 min removed unbroken cells. The cell membranes were pelleted out of the supernatant by centrifugation at 46,000 × *g* for 60 min. They were washed with one volume of 50 mM sodium phosphate buffer (pH 7.0) recentrifuged, and suspended in one-half volume of phosphate buffer (pH 7.0) at a protein concentration of 2 mg/ml. Labeled penicillin (20 μl) in phosphate-buffered saline was added to 200-μl aliquots of whole cells at a density of 10^9 cells per ml or to 200-μl aliquots of membranes. The reaction was stopped after 15 min of incubation in a 35°C water bath by the addition of 5 μl of unlabeled benzylpenicillin (100 mg/ml) and 20 μl of 20% Sarkosyl. After 30 min of incubation at room temperature, the Sarkosyl-insoluble fraction was removed by centrifugation at 128,000 × *g* for 30 min in a Beckman Airfuge (Beckman Instruments, Inc., Fullerton, Calif.)

Gel electrophoresis and detection of PBPs. PBPs were identified in the Sarkoysl-soluble fractions of whole cells and isolated membranes with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography by methods described previously (4). The exposure time for [^{3}H]penicillin and presensitized Kodak X-Omat AR film at −76°C was 7 days. The amount of binding of [^{3}H]penicillin to PBPs was estimated from fluorograms by densitometric scanning with a Joyce-Loebl microdensitometer. Integration of peaks was performed with the aid of a Zeiss MOP 3 image analyzer. The criteria for the saturation of and 50% binding to PBPs were those previously reported (4).

RESULTS

Penicillin susceptibility. Table 1 shows the minimum concentrations of penicillin that were completely inhibitory to growth (0.4 nmol/ml or 0.15 μg/ml) and that were bactericidal (3.1 nmol/ml or 1.1 μg/ml). Growth inhibition was detectable but incomplete at 0.2 nmol/ml. Although 0.8 and 1.6 nmol/ml reduced the spirochete count to undetectable levels, 10 or more organisms per ml apparently survived and were able to produce infections in mice. Borreliae exposed to 3.1 nmol/ml appeared to be either killed or rendered noninfectious for this mouse strain.

Electron microscopy. *Borrelia* cultures were treated with 0.03, 0.3, or 3.1 nmol of benzylpenicillin per ml for 10 h and were examined by phase contrast light microscopy. There was no apparent morphological effect on organisms exposed to 0.03 and 0.3 nmol/ml, but approximately one-half of the cells in the culture containing 3.1 nmol/ml showed one or two large blebs associated with their surfaces. Untreated borreliae in late-log-phase growth (Fig. 1) and parallel cultures of borreliae exposed to 3.0 nmol of benzylpenicillin per ml for approximately one to two generations (Fig. 2 and 3) were examined by electron microscopy. Figure 1 shows longitudinal and cross sections of untreated borreliae. An axial filament runs the length of a cell. The bundles of small, electron dense fibers located eccentrically at the periphery of protoplasmic cylinders are axial filaments in cross section. The thin sections of treated borreliae revealed numerous large ovoid and spherical forms that were either separate from or still associated with recognizable protoplasmic cylinders (Fig. 2). In some cross sections two membranes surrrounded diffuse cytoplasmic material or remnants of the cylinder (Fig. 3). Figure 4 shows the apparent rupture of the protoplasmic cylinder at the location of the axial filament. This was the most common site for the large bulges to occur. We did not observe extremely long spirochetes or rod-shaped forms. An additional finding was that of smaller spherical blebs of 80- to 100-nm diameter in electron micrographs of both the untreated and treated cultures; these smaller

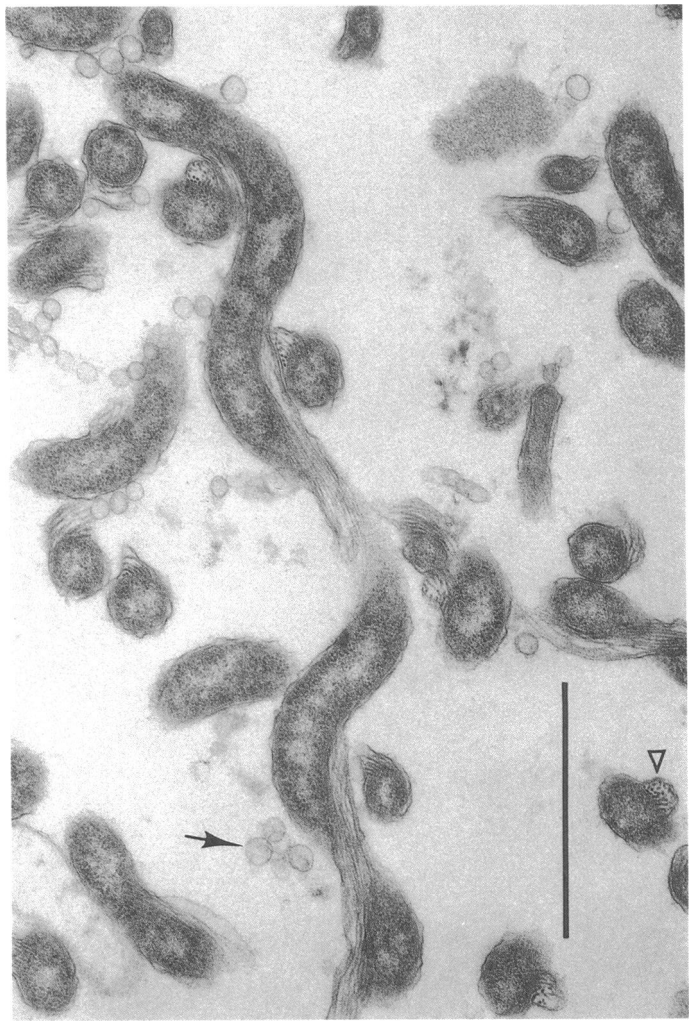

FIG. 1. Electron photomicrograph of a thin section of untreated *B. hermsii* cells. Longitudinal and cross sections are illustrated. The arrow indicates a small membranous bleb, and the open triangle indicates an axial filament in cross section. Bar = 1 μm.

blebs were considerably more common in the treated culture (Figs. 1 and 2).

Binding of labeled penicillin to PBPs of *B. hermsii*. Figure 4 shows the PBPs when whole cells of *B. hermsii* strain HS1 were incubated with [^{3}H]penicillin. The figure also shows the location of the five PBPs in relation to Coomassie blue-stained proteins in the Sarkosyl-soluble fraction of whole cells that was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The apparent subunit molecular weights of the PBPs, when assessed by the addition of molecular weight standards to the gel, were 90,000 (PBP 1), 64,000 (PBP 2), 60,000 (PBP 3), 30,000 (PBP 4), and 29,000 (PBP 5). The same number and sizes of PBPs were noted when isolated membranes were used in place of whole cells (data not shown). No bands were seen when the Sarkosyl-insoluble fractions of whole cells and isolated membranes were examined by gel electrophoresis and fluorography.

Binding of [^{3}H]penicillin to the five PBPs did not detectably occur when cells or membranes were incubated with unlabeled penicillin at a concentration of 10 μmol/ml, boiled for 5 min, or solubilized in 1% sodium dodecyl sulfate in phosphate-buffered saline before exposure to labeled penicillin. Additional PBPs that may have bound penicillin unstably were not detected when the incubation period for membranes and labeled penicillin was shortened to 30 s or to 2 min. To assess the release of labeled penicillin from PBPs, a 1,000-fold excess of unlabeled penicillin was added to the [^{3}H]penicillin-mem-

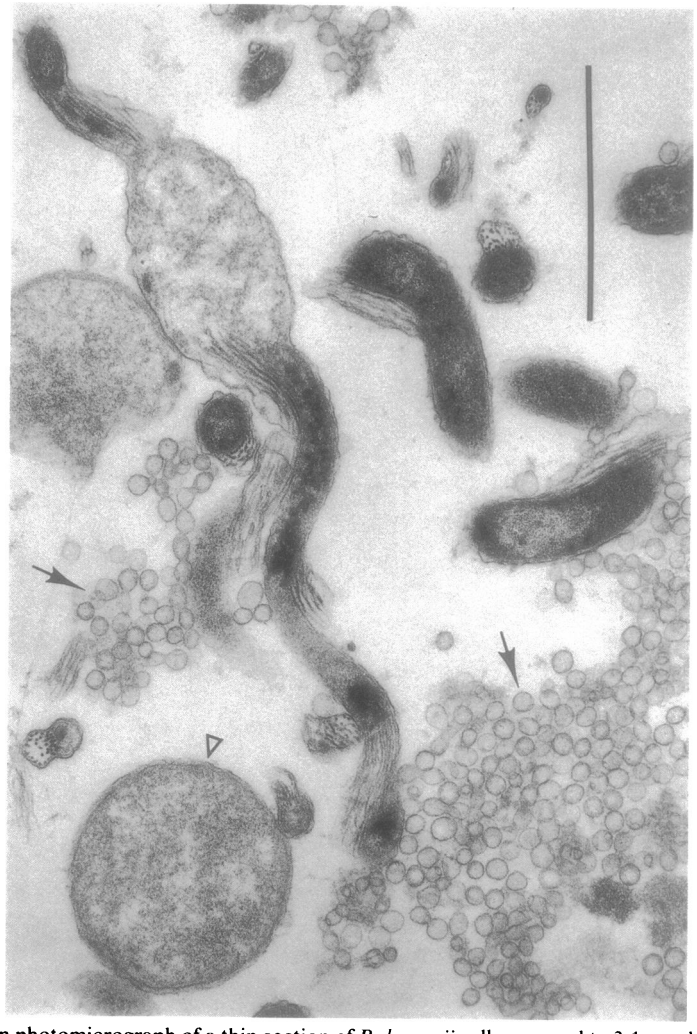

FIG. 2. Electron photomicrograph of a thin section of *B. hermsii* cells exposed to 3.1 nmol of benzylpenicillin per ml for 10 h. Large ovoid and spherical structures are illustrated; an open triangle points to one example. Arrows indicate small membranous blebs. Bar = 1 μm.

brane reaction mixture. There was no detectable release of label from any PBP when excess unlabeled penicillin was present for up to 40 min.

The 50% binding concentration of [^{3}H]penicillin for the five PBPs in whole cells were 0.3, 0.9, 0.8, 0.1, and 0.1 nmol/ml, respectively. At a concentration of 3.1 nmol/ml, the percentages of total [^{3}H]penicillin bound to individual PBPs in whole cells were 56, 7, 19, 13, and 5% respectively.

DISCUSSION

We examined three aspects of the exposure of *B. hermsii* to penicillin: the in vitro antibiotic susceptibility of the strain, the morphological response of these spirochetes to penicillin, and the PBPs in the cells. *B. hermsii* strain HS1 was inhibited in its growth and killed by benzylpenicillin at micromolar concentrations; the predominant morphological response at the minimum bactericidal concentration was the formation of spheroplasts and small blebs; and the minimum inhibitory concentration was within a factor of five of the 50% binding concentrations of labeled penicillin for the five PBPs.

The in vivo effectiveness of penicillin for borrelial as well as other spirochetal infections has been well documented (3, 5, 9, 24). In vitro susceptibility assessments of β-lactam and other antibiotics have been performed on the in vitro-cultivable treponemes and oral borreliae (1, 2, 10), but they apparently have not been per-

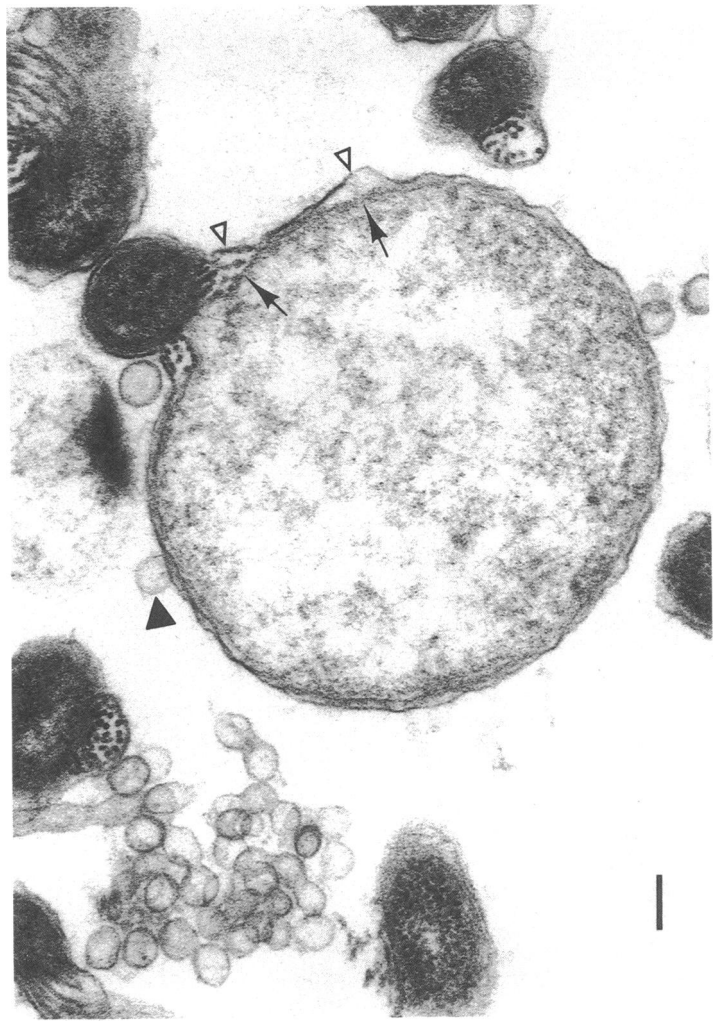

FIG. 3. Electron photomicrograph of a thin section of a *B. hermsii* cell exposed to 3.1 nmol of benzylpenicillin per ml for 10 h. The arrows indicate the inner membrane, and the open triangles indicate the outer membrane. A closed triangle points to a small bleb associated with the membranes. Bar = 0.1 μm.

formed on relapsing fever borreliae. A comparison of our findings with studies of in vitro-cultivable treponemes and oral borreliae shows that *B. hermsii* resembles the majority of other spirochetes in being inhibited in its growth by 0.1 to 1.0 nmol of benzylpenicillin per ml (1). The finding of a minimum bactericidal concentration no more than eightfold higher than the minimum inhibitory concentration is similar to that of Abramson and Smibert in the case of *Treponema vincenti* (2).

Benzylpenicillin at its minimum bactericidal concentration induced formation of large spherical structures. These structures were bounded by one or both cellular membranes and, in some thin sections, appeared to contain material from disrupted protoplasmic cylinders. Joseph et al. found similar structures among free-living spirochetes exposed to 5 mg of penicillin per ml (17). Although we have not assessed the amount of peptidoglycan in these large spherical structures, they are consistent in appearance with spheroplasts (20, 29). The presence of two membranes in the envelopes of these spherical structures distinguishes them from "spherical bodies," which occur when some spirochetes are suspended in hyperosmotic media (28). Spherical bodies represent separations of the outer membrane (envelope) from the underlying peptidoglycan-cytoplasmic membrane complex (28).

A prominent electron microscopic finding was the abundance of small membranous blebs or vesicles in the penicillin-treated culture. Blebbing of the outer membrane is said to occur

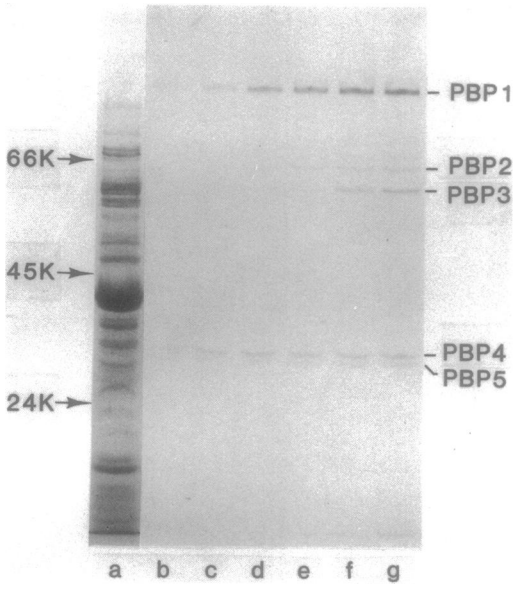

FIG. 4. Fluorograms of PBPs and Coomassie blue-stained proteins of *B. hermsii* cells. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the Sarkosyl-soluble fraction of whole cells was from top to bottom. Track a contains proteins stained with Coomassie blue R-250. Tracks b through g contain fluorograms of PBPs. The following concentrations (nanomoles per milliliter) of [^{3}H]penicillin were incubated with the preparations: b, 0.012; c, 0.05; d, 0.2; e, 0.4; f, 0.8; and g, 1.6. The molecular weight standards (Sigma Chemical Co., St. Louis, Mo.) were bovine serum albumin (66K), ovalbumin (45K), and trypsinogen (24K).

when spirochetes are under "adverse conditions" (16). An examination of the mechanisms of blebbing of gram-negative bacteria provided possible explanations for this phenomenon in the less extensively studied spirochetes. Outer membranes blebs of approximately the same size as noted in *B. hermsii* cultures are produced by *Escherichia coli* (15). Bleb production by *E. coli* increases under conditions that slow protein synthesis (19). Wensink and Witholt have proposed, in the case of *E. coli*, that outer membrane vesicles form when the outer membrane expands faster than the underlying peptidoglycan layer (30). If, as appears to be the case for spirochetes (21), the association between the outer membrane and peptidoglycan is normally weak, then spirochetes may form many blebs as a result of inhibition of peptidoglycan synthesis.

A possible consequence of penicillin-induced membrane vesicle formation is the Jarisch-Herxheimer reaction, the systemic reaction that often follows treatment of relapsing fever with antibiotics (22). Some investigators have demonstrated endotoxin-like material in the sera of humans and animals with borrelial infections (6, 12, 31) and in the sera of humans with Jarisch-Herxheimer reaction after treatment with penicillin for secondary syphilis (13). A release of numerous blebs containing such material conceivably could precipitate the Jarisch-Herxheimer reaction. Alternatively, the blebs may be the "non-endotoxin particulate pyrogen" described by Butler et al. (7). Further studies are planned on the nature of the small blebs and their possible association with the Jarisch-Herxheimer reaction.

We identified five PBPs in the Sarkosyl-soluble fraction of *B. hermsii*. The lack of binding of labeled penicillin to proteins of boiled membranes, sodium dodecyl sulfate-solubilized membranes, and membranes pretreated with excess unlabeled penicillin suggested that we had detected true PBPs (8). The similar 50% binding concentrations of labeled penicillin for the five PBPs in whole cells and for the minimum inhibitory concentration indicated that one or more of the PBPs were probably penicillin targets. The narrow range of 50% binding concentrations for the five PBPs, however, makes the assignment of any PBP to a certain function in peptidoglycan synthesis or modification impossible at this time. We can, however, compare the apparent molecular weights of *B. hermsii* PBPs with those of PBPs of other genera of bacteria. The number and the range of molecular weights of *B. hermsii* PBPs are similar to those found in other noncocal bacteria (14, 25). What is unique is the abundant binding of penicillin to the largest PBP (PBP 1) in *B. hermsii*. In view of the phylogenetic distance of the spirochetes from the purple photosynthetic group of eubacteria, which include the *Enterobacteriaceae*, and from gram-positive bacteria (11, 23), further study of spirochetal PBPs and their functions may be of interest.

LITERATURE CITED

1. **Abramson, I. J., and R. M. Smibert.** 1971. Inhibition of growth of treponemes by antimicrobial agents. Br. J. Vener. Dis. **47**:407–412.
2. **Abramson, I. J., and R. M. Smibert.** 1971. Bactericidal activity of antimicrobial agents for treponemes. Br. J. Vener. Dis. **47**:413–418.
3. **Augustine, D. L., D. Weinman, and J. McAllister.** 1944. Rapid and sterilizing effect of penicillin sodium in experimental relapsing fever infections and its ineffectiveness in the treatment of trypanosomiasis (*Trypanosoma lewisi*) and toxoplasmosis. Science **99**:19–20.
4. **Barbour, A. G.** 1981. Properties of penicillin-binding proteins in *Neisseria gonorrhoeae*. Antimicrob. Agents Chemother. **19**:316–322.
5. **Brause, B. D., J. S. Borges, and R. B. Roberts.** 1976. Relative efficacy of clindamycin, erythromycin, and penicillin in treatment of *Treponema pallidum* in skin syphilomas of rabbits. J. Infect. Dis. **134**:93–96.
6. **Bryceson, A. D. M., K. E. Cooper, D. A. Warrell, P. L. Perine, and E. H. O. Parry.** 1972. Studies of the mechanism of the Jarisch-Herxheimer reaction in louse-borne

relapsing fever: evidence for the presence of circulating *Borrelia* endotoxin. Clin. Sci. **43**:343–354.

7. **Butler, T., P. Hazen, C. K. Wallace, S. Awoke, and A. Habte-Michael.** 1979. Infection with *Borrelia recurrentis*: pathogenesis of fever and petechiae. J. Infect. Dis. **140**:665–675.
8. **Dougherty, T. J., A. E. Koller, and A. Tomasz.** 1980. Penicillin-binding proteins of penicillin-susceptible and intrinsically resistant *Neisseria gonorrhoeae*. Antimicrob. Agents Chemother. **18**:730–737.
9. **Eagle, H., and H. J. Magnuson.** 1944. The therapeutic efficacy of penicillin in relapsing fever infections in mice and rats. Public Health Rep. **59**:583–588.
10. **Eagle, H., and A. D. Musselman.** 1944. The spirocheticidal action of penicillin in vitro and its temperature coefficient. J. Exp. Med. **80**:493–505.
11. **Fox, G. E., E. Stackebrandt, R. B. Hespell, J. Gibson, J. Maniloff, T. A. Dyer, R. S. Wolfe, W. E. Balch, R. S. Tanner, L. J. Magrum, L. B. Zablen, R. Blakemore, R. Gupta, L. Bonen, B. J. Lewis, D. A. Stahl, K. R. Luehrsen, K. N. Chen, and C. R. Woese.** 1980. The phylogeny of prokaryotes. Science **209**:457–463.
12. **Galloway, R. E., J. Levin, T. Butler, G. B. Naff, G. H. Goldsmith, H. Saito, S. Awoke, and C. K. Wallace.** 1977. Activation of protein mediators of inflammation and evidence for endotoxinemia in *Borrelia recurrentis* infection. Am. J. Med. **63**:933–938.
13. **Gelfand, J. A., R. J. Flin, F. W. Berry, and M. M. Frank.** 1976. Endotoxemia associated with the Jarisch-Herxheimer reaction. N. Engl. J. Med. **295**:211–213.
14. **Georgopapadakou, N. H., and F. Y. Liu.** 1980. Penicillin-binding proteins in bacteria. Antimicrob. Agents Chemother. **18**:148–157.
15. **Hoekstra, D., J. W. Van Der Laan, L. DeLeij, and B. Witholt.** 1976. Release of outer membrane fragments from normally growing *Escherichia coli*. Biochim. Biophys. Acta **455**:889–899.
16. **Johnson, R. C.** 1977. The spirochetes. Annu. Rev. Microbiol. **31**:89–106.
17. **Joseph, R., S. C. Holt, and E. Canale-Parola.** 1973. Peptidoglycan of free-living anaerobic spirochetes. J. Bacteriol. **115**:426–435.
18. **Kelly, R.** 1971. Cultivation of *Borrelia hermsii*. Science **173**:443–444.
19. **Knox, K. W., M. Vesk, and E. Work.** 1966. Relation between excreted lipopolysaccharide complexes and surface structures of a lysine-limited culture of *Escherichia coli*. J. Bacteriol. **92**:1206–1217.
20. **Martin, H. H.** 1963. Bacterial protoplasts—a review. J. Theor. Biol. **5**:1–34.
21. **Morioka, H., H. Ozasa, T. Kishida, Y. Yokota, and A. Suganuma.** 1979. The peptidoglycan layer of the Reiter treponeme as revealed by thin sectioning and freeze-etching techniques. J. Electron Microsc. **28**:189–192.
22. **Sanford, J. P.** 1976. Relapsing fever—treatment and control, p. 389–394. *In* R. C. Johnson (ed.), The biology of the parasitic spirochetes. Academic Press Inc., New York.
23. **Schleifer, K. H., and O. Kandler.** 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol. Rev. **36**:407–477.
24. **Schuhardt, V. T.** 1952. Treatment of relapsing fever with antibiotics. Ann. N.Y. Acad. Sci. **55**:1209–1221.
25. **Spratt, B. G.** 1977. Properties of the penicillin-binding proteins of *Escherichia coli* K12. Eur. J. Biochem. **72**:341–352.
26. **Stoenner, H. G.** 1974. Biology of *Borrelia hermsii* in Kelly medium. Appl. Microbiol. **28**:540–543.
27. **Thompson, R. S., W. Burgdorfer, R. Russell, and B. J. Francis.** 1969. Outbreak of tick-borne relapsing fever in Spokane County, Washington. J. Am. Med. Assoc. **210**:1045–1050.
28. **Umemoto, T., and I. Namikawa.** 1980. Electron microscopy of the spherical body of oral spirochetes in vitro. Microbiol. Immunol. **24**:321–334.
29. **Webull, C.** 1968. The morphology of protoplasts, spheroplasts, and L-forms, p. 62–73. *In* L. B. Guze (ed.), Microbial protoplasts, spheroplasts, and L-forms. The Williams & Wilkins Co., Baltimore.
30. **Wensink, J., and B. Witholt.** 1981. Outer membrane vesicles released by normally growing *Escherichia coli* contain very little lipoprotein. Eur. J. Biochem. **116**:331–335.
31. **Wright, D. J. M., and D. F. Woodrow.** 1980. Terminal changes in mice experimentally infected with *Borrelia duttoni*. J. Pathol. **130**:83–90.