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ISOLATION, PRELIMINARY CHEMICAL CHARACTERIZATION, AND BIOLOGICAL ACTIVITY OF *Borrelia burgdorferi* PEPTIDOGLYCAN[1]

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Peptidoglycan (PG), an essential cell wall polymer of most bacteria, has been isolated from many species of spirochetes. Our interest in the host response to *Borrelia burgdorferi* led us to isolate and characterize its PG. Extracted cells were solubilized with warm 1% SDS followed by digestion with proteases. Amino acid analysis of the isolated PG demonstrated the presence of alanine, glycine, glutamic acid, and ornithine as occurs in other spirochetes and bacteria. Intense erythematous reactions were observed after *id* injection of 10 μg of PG into normal human skin. PG was not mitogenic for human peripheral blood mononuclear cells. Murine splenocytes of certain strains responded to the PG, but only at concentrations of 25 μg/ml or more. PG stimulated macrophages to produce interleukin 1. Sixteen μg of PG injected *iv* into rabbits produced biphasic fevers. These observations on the *in vitro* and *in vivo* activities associated with the cellular components of the *B. burgdorferi* spirochete give further insight to how a small number of invading organisms can cause a multisystemic disease such as Lyme disease.

Lyme disease, first recognized as a focal outbreak of arthritis in Old Lyme, Connecticut, is caused by the bite of an *Ixodes* tick infected with the spirochete *Borrelia burgdorferi* (1,2). This spirochete has been isolated from blood, skin lesions, spinal fluid, and synovial fluid from patients with Lyme disease (2,3,4). Evidence to date indicates that a relatively few spirochetes in the infected host produce chronic, systemic disease (5,6). This suggests that the spirochetes possess or produce a potent mediator that can amplify the effect of this small number of invading organisms. We have previously isolated a lipopolysaccharide (LPS) from this organism that possesses all the chemical characteristics and biologic activities of other LPS (7). Another bacterial component with potent biological activities which might account for the pathogenicity of a small number of spirochetes is peptidoglycan (PG). For this reason we wished to characterize the peptidoglycan from *B. burgdorferi* and compare it to the PG from related species (8).

Peptidoglycan is an important component of bacterial cells. It is an essential cell wall polymer and has been isolated from many species of spirochetes (8). Spirochetal PG is associated with the cytoplasmic membrane and it is thought to be involved in maintaining cell rigidity and shape (9). It also serves to confer the coiled configuration of the spirochete (9). In most species studied, PG consists of a glycan backbone with alternating β 1-4-linked residues of N-acetyl-D-glucosamine and muramic acid (10,11).

The biological activities of PG are diverse and complex (12). Its immunopotentiating activities have made it the subject of intense scrutiny. Some of the varied properties of PG include induction of antibody formation,

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immunomodulation, complement activation, release of mediators, induction of inflammation, and increased macrophage phagocytosis (10).

Many of the symptoms of Lyme disease could be explained by the host response to spirochetal PG. These include fever, arthritis, erythema chronicum migrans, and malaise. Our interest in the host response in Lyme disease led us to study its PG. We therefore extracted and characterized the PG from *Borrelia burgdorferi*.

MATERIALS AND METHODS

Materials. Pyrogen free water and pyrogen free saline were obtained from Travenol (Deerfield, IL) and were used to prepare all solutions. Fetal calf serum (FCS, lot no. 1111610; endotoxin level <0.008 ng/ml) was obtained from HyClone Labs (Logan, UT). Sterile pyrogen free syringes and needles were obtained from Becton-Dickinson (Rutherford, NJ). All plasticware was obtained from Falcon (Oxnard, CA). All tissue culture media were obtained from Flow Laboratories (McLean, VA). All other reagents were of analytical grade or better and were obtained from Sigma (St. Louis, MO) or Fisher (Springfield, NJ).

Cell culture. The murine macrophage cell line P388D1 was grown in RPMI 1640 containing 10% heat-inactivated FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml). The Shelter Island isolate of the Lyme disease spirochete *Borrelia burgdorferi* was grown and passaged as described (7). Before use, spirochetes were washed in sterile pyrogen free saline three times by resuspension after centrifugation at 9,000 × g at 4°C for 20 min.

Preparation of spirochete lipopolysaccharide (LPS). LPS was isolated from *Borrelia burgdorferi* by the petroleum-ether:chloroform:liquid-phenol (PCP) extraction procedure as previously described (7). We have since modified the procedure to include a final treatment step with proteinase-K to remove any residual protein. Twenty five μg of proteinase-K was added to the LPS (1 mg/ml) for 5 hrs at 37°C. The LPS was then dialyzed (3-4,000 dalton cut off, Spectrum Medical, Inc., Los Angeles, CA) in phosphate-buffered saline (PBS). When electrophoresed on 15% SDS-PAGE gels and stained for protein with silver (7), there were no silver staining protein bands. Also, no protein was detected in the LPS when assayed by the method of Spector (13).

Preparation of spirochete peptidoglycan (PG). We modified the method of Umemoto *et al.* to extract the peptidoglycan (14). Briefly, after the second extraction step of the isolation of the LPS (7), we extracted the recovered pellet in 1% SDS for 18 hrs at 37°C. This mixture was centrifuged at 110,000 × g for 60 min and the recovered pellet was resuspended in 1% SDS. After incubation, again at 37°C for 18 hrs, and another centrifugation the pellet was washed with 6 M urea to remove the residual SDS. The pellet was put into sterile pyrogen free distilled water and the mixture was centrifuged at 1,500 × g to remove debris. This supernatant was centrifuged at 110,000 × g and the pellet lyophilized. The resulting material was suspended in .01 M Tris pH 7.4 (14.5 ml) and incubated with trypsin (1.46 mg) and stirred for 18 hrs at 37°C. This mixture was washed twice with sterile pyrogen free distilled water by centrifugation at 110,000 × g for 90 min. The pellet was resuspended in the Tris buffer (14.5 ml) and Pronase P (1.46 mg) was added and the mixture was stirred for 18 hrs at 37°C. The pellet obtained by centrifugation at 110,000 × g for 90 min was washed twice with sterile pyrogen free distilled water. The final pellet was lyophilized and designated PG.

Preparation of radioiodinated albumin. Bovine serum albumin (BSA) was radioiodinated using Iodo-Beads (Pierce Chem. Co., Rockford, IL) and Iodine-125 (1 mCi, >350 mCi/ml; New England Nuclear, Boston, MA) according to the method of Markwell (15). The iodinated albumin was passed over a Dowex-1-chloride (Sigma) column to remove unbound iodine. The [^{125}I]-albumin ([^{125}I]-BSA) had a specific activity of 1 μCi/μg.

Measurement of vascular permeability. Preparation and handling of rabbits and intradermal injections of experimental agents and controls were as described in detail previously (16). Briefly, test materials were injected intradermally in a volume of 0.1 ml into shaved rabbit skin at various times before sacrifice. Fifteen minutes before sacrifice by lethal injection (T61: Hoechst Corp., Somerville, NJ), [^{125}I]-BSA was injected into the marginal ear vein. The skin on the backs was removed, and the blood in the large veins was expelled by pushing it to the edge. The skin was frozen at -70°C and the lesions were punched out with a 1.5 cm steel punch. Radioactivity in the tissue sample was counted in a well type gamma counter. A sample of blood removed 5 min before sacrifice was used to measure the amount of [^{125}I]-BSA in 1 μl of serum. All agents were diluted in pyrogen free saline and sterilized by filtration (0.22 μm; Millipore, Bedford, MA) before injection.

Interleukin 1 (IL-1) assay: Thymocyte proliferation assay. Samples were assayed for lymphocyte activation factor (LAF) activity as a measure of IL-1 activity with thymocytes from 4 to 8 week old BALB/c mice, as described (16). For all assays, significance of differences was assessed by Student's *t* test.

Mitogenesis assays. PG and control fractions were assayed for mitogenic activity with murine splenocytes as described in detail previously (7). Spleens from four-to eight-week-old C3H/HeJ, BALB/c, AKR, and C57BL/6 mice were used. PG and control fractions were assayed for mitogenic activity using human peripheral blood mononuclear cells as described in detail previously (7).

Pyrogen assay. New Zealand albino rabbits weighing 4-6 kg. were used as previously described in detail (7). Materials to be assayed and control fractions were diluted in pyrogen free saline. A rise in temperature of <0.6°C was not considered significant.

Detection of endotoxin. A limulus amebocyte lysate (LAL) assay using a chromogenic substrate was employed as described previously (7). The kit was obtained from M.A. Bioproducts (Walkersville, MD) and used according to the manufacturer's instructions.

Characterization of the isolated PG. The amino acid composition of the isolated PG was analyzed on a PICO-TAG amino acid analysis system (Waters Assoc., Milford, MA). Samples were hydrolysed with 6 N-HCl in a sealed tube at 105°C for 18 hrs. The manufacturer's amino acid standard was spiked with ornithine in order to determine its elution time.

RESULTS

Preparation and chemical characterization of spirochete peptidoglycan. Peptidoglycan (PG) was extracted from *Borrelia burgdorferi* by a modification of the technique described by Umemoto and coworkers (14). Spirochetes were extensively washed and extracted with PCP to remove the LPS. This LPS has been described previously (7), and has characteristics of a "rough" LPS and therefore partitions to the organic phase. The pellet that remains contains cellular debris and it is with this pellet that we started the extraction of the PG. This modification of the Umemoto technique allowed us to separate the LPS from the PG early in the extraction process. From the amount of material recovered we estimate that the PG accounted for approximately 0.01% of the dry weight of the spirochete.

Many of the biologic properties of the PG and LPS are shared (10). We employed a chromogenic LAL test to determine the degree of LPS contamination in the PG preparation. This is an important consideration, since any contaminating LPS activity could also give positive results in our assays. Any LPS from outside sources would also give false positive results. Analysis of the purified PG showed a negligible amount of LPS (<0.009 ng/mg PG) in our PG preparation.

The amino acid composition of the PG is shown in Table 1. The relative molar amounts of the predominant amino acids involved in the PG of *Borrelia burgdorferi*, glycine, alanine, ornithine, and glutamic acid were approximately 1.5:1:1:1. Serine, leucine, and lysine were detected, but accounted for a very small percentage of the amino acids and are probably not associated with the peptide bridge of the PG.

Mitogenic activity of spirochete PG. The mitogenic activity of the PG was first determined in spleen cell cultures of several strains of mice. The effect of PG on *in vitro* incorporation of [^{3}H]-thymidine by murine splenocytes is shown in Table 2. All strains of mice responded to the PG, with the response of some being

Table 1
Relative molar ratios of amino acids of *B. burgdorferi* peptidoglycan

Amino Acid	Relative Molar Ratio[a]
Alanine	1.43
Glycine	1.00
Ornithine	0.95
Glutamic Acid	0.91
Serine	0.18
Proline	0.12
Lysine	0.11

[a]Expressed as relative molar ratios to glycine.

Table 2
The peptidoglycan has a mitogenic effect on murine splenocytes of certain strains

	Tritiated Thymidine Incorporation (DPM ± SEM)			
	Strain			
Concentration (μg/ml)	C3H/HeJ	BALB/c	AKR	C57BL/6
0	4,152 ± 622	10,351 ± 525	4,006 ± 288	6,000 ± 467
1	3,944 ± 335	12,463 ± 1,246	5,034 ± 503	9,511 ± 952[a]
5	4,518 ± 632	14,292 ± 1,479	6,404 ± 810[a]	19,256 ± 2,902[c]
10	4,855 ± 485	12,271 ± 1,727	8,672 ± 687[c]	24,282 ± 2,428[c]
25	6,396 ± 350[a]	14,581 ± 3,353	9,725 ± 914[c]	25,884 ± 3,346[c]
50	6,911 ± 1,105[b]	22,484 ± 1,913[c]	15,192 ± 2,122[c]	35,161 ± 7,735[a]
100	8,562 ± 250[c]	26,471 ± 1,805[c]	19,376 ± 408[c]	47,201 ± 4,720[c]

[a] $p < 0.01$ as compared to control.
[b] $p < 0.02$ as compared to control.
[c] $p < 0.001$ as compared to control.

more sensitive than others. C3H/HeJ and BALB/c mice responded, but only with high concentrations of PG. The AKR and C57BL/6 mice responded with a doubling of the background at concentrations in the 5-10 μg/ml range.

The mitogenic activity of the spirochete PG was also determined by using normal human peripheral blood mononuclear cells. The PG had no effect on the uptake of [^{3}H]-thymidine by the mononuclear cells. When some preparations of mononuclear cells were further purified by rosetting to isolate T and B cells, these individual populations of lymphocytes did not respond to the PG even at concentrations of >500 μg/ml (data not shown).

Stimulation of murine macrophages to produce interleukin 1 (IL-1). When PG was added to the murine macrophage cell line P388D1 the supernatants collected after 24 hrs contained LAF activity that was detected in the murine thymocyte proliferation assay. Concentrations of PG as small as 0.5 μg/ml were capable of stimulating the P388D1 cells to release interleukin 1 (data not shown).

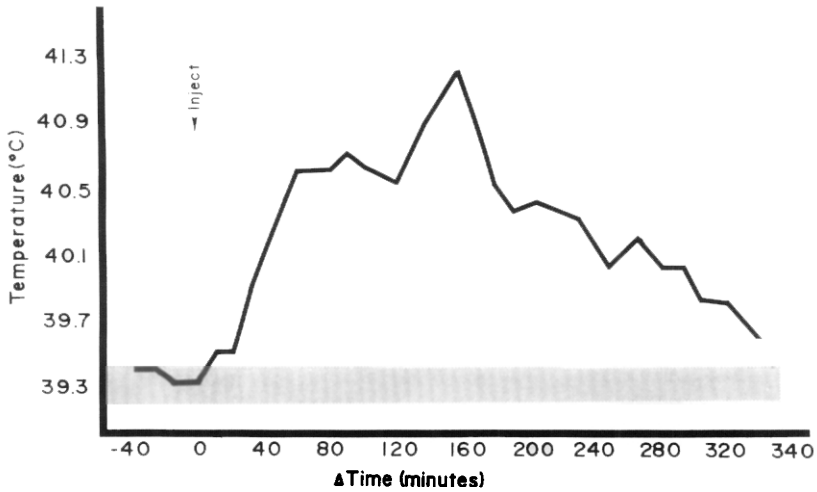

Figure 1. The peptidoglycan is pyrogenic in rabbits. Sixteen μg of spirochete PG were injected *iv* and the rectal temperature was recorded at the times indicated. The shaded area represents the background temperature of control rabbits.

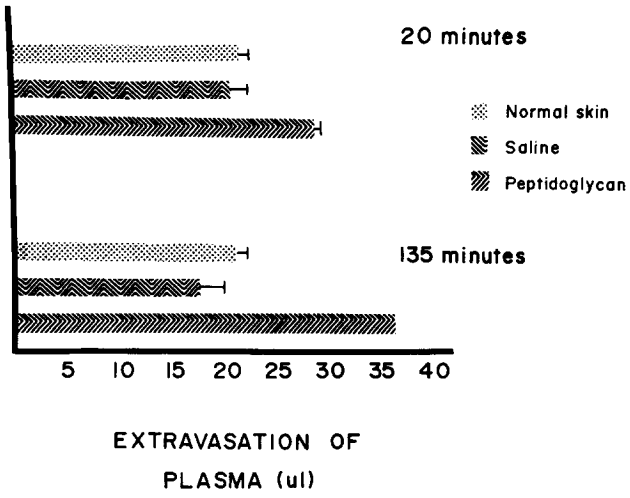

Figure 2. The peptidoglycan causes an increase in vascular permeability. Samples as indicated were injected subcutaneously into shaved rabbit backs. The amount of vascular leakage was quantified by measuring the amount of [^{125}I]-BSA in the injection sites.

Pyrogenicity of spirochete PG. To measure pyrogenicity, we injected New Zealand albino rabbits *iv* with the spirochete PG and measured rectal temperatures. Figure 1 shows the results of a representative experiment in which 16 µg were injected. As can be seen, a classic biphasic fever profile was observed.

***In vivo* inflammatory effects of spirochete PG.** Two experimental models were chosen to investigate the *in vivo* inflammatory properties of the spirochete PG. In the first model PG was injected intradermally into shaved rabbit backs and increases in vascular permeability were measured by quantifying the

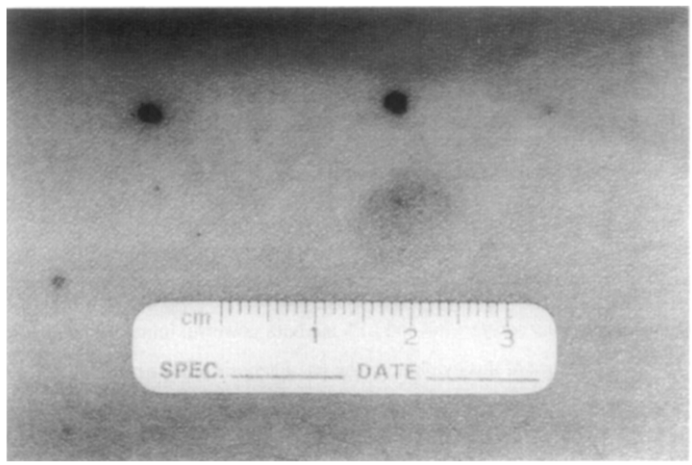

Figure 3. The peptidoglycan causes an intense skin reaction when injected intradermally. Ten µg of PG were injected intradermally into the forearm of a normal volunteer (right side). Saline was injected into a control site (left side). The sites were observed and measured for the duration of the inflammatory reaction (approximately 72 hrs). The photograph was taken 24 hrs after the injection.

amount of [^{125}I]-BSA leakage at the injection site. As can be seen in Figure 2 the change in vascular permeability could be seen in as little as 20 min, with leakage still occurring at 135 min. In this representative experiment 15 µg of PG were used.

In the second model, PG was injected subcutaneously into the forearm of a human volunteer (GB). Ten µg (in 25 µl sterile pyrogen-free saline) were injected and the area was observed for any changes. A skin reaction characterized by erythema and induration was observed. The reaction started a few minutes after injection and reached a peak by 36 hrs and then subsided. The lesion disappeared after a few days and no permanent damage was seen. Figure 3 shows the skin lesion at 24 hrs. At this time the lesion was red, indurated, and warm to the touch. The site on the left was injected with saline (25 µl).

DISCUSSION

In this paper we report on the isolation and characterization of a PG extracted from the Lyme disease spirochete *Borrelia burgdorferi*. The isolation procedure was fast and separated the PG from any contaminating spirochetal LPS.

The amino acid analysis of the PG revealed the major amino acids to be alanine, glycine, glutamic acid, and ornithine. These results are in agreement with Yanagihara *et al.* (8), who isolated PG from a similar spirochete (*Borrelia hermsi*). They found the major amino acids to be alanine, glycine, and glutamic acid, and ornithine. In a preliminary examination of *B. burgdorferi* Johnson *et al.* (17) reported that the PG diamino amino acid is ornithine.

The biologic activities associated with this PG are similar to those described for other isolated PG. Pyrogenicity is a major activity of PG (10,18). Our PG was a potent pyrogen, and it is unlikely that the activity was due to contaminating endotoxin in our preparations since the LAL test was negative. Also, the amount of LPS present in the injected PG (<0.14 pg) could not account for the fever observed. Stimulation of macrophages by PG to produce IL-1 has been observed by several groups (19,20). A biphasic fever may result from both direct pyrogenicity and the *in vivo* induction of IL-1 by PG (7). The inflammatory properties of PG include increases in capillary permeability (21). This may be caused by the ability of PG to induce the release of endogenous mediators of inflammation (*e.g.*, histamine) (10). Our experiments at this time cannot answer this question. Acute inflammation of the skin has been observed as a major activity of PG in both man and animals (10,18).

Many of the symptoms of Lyme disease could be explained by the activities of the PG. Fever, the characteristic skin rash erythema chronicum migrans, arthritis, and malaise (22) could all be caused by direct action of the PG. Taken with our data on the isolation and biologic characterization of the LPS from this spirochete it is easy to speculate that both these cellular components contribute to the multisystemic course of Lyme disease. That both agents induce the production of IL-1 could contribute to the "biologic amplification" of a small number of invading spirochetes.

In conclusion, the role of PG and LPS in discussions of the pathogenesis of Lyme disease must now be taken into consideration. That *B. burgdorferi* PG and LPS are both powerful immunomodulators and stimulators of IL-1 gives credence to the importance of this cytokine in the pathogenesis of Lyme disease.

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REFERENCES

1. Habicht, G.S., Beck, G., and Benach, J.L. (1987) Scientific American. July 257, 78–83.
2. Steere, A.C., Grodzicki, R.L., Kornblatt, A.N., Craft, J.E., Barbour, A.G., Burgdorfer, W., Schmid, G.P., Johnson, E., and Malawista, S.E. (1983) N. Engl. J. Med. 308, 733–740.
3. Benach, J.L., Bosler, E.M., Hanrahan, J.P., Coleman, J.L., Habicht, G.S., Bast, T.F., Cameron, D.J., Ziegler, J.L., Barbour, A.G., Burgdorfer, W., Edelman, R., and Kaslow, R.A. (1983) N. Engl. J. Med. 308, 740–742.
4. Snyderman, D.R., Schenkein, D.P., Berardi, V.P., Lastavica, C.C., and Pariser, K.M. (1986) Ann. Intern. Med. 104, 798–800.
5. Johnson, Y.E., Duray, P.A., Steere, A.C., Kashgarian, M., Buza, J., Malawista, S.E., and Askenase, P.W. (1985) Am. J. Pathol. 118, 26–34.
6. Kornblatt, A.N., Steere, A.C., and Brownstein, D.G. (1984) Infect. Immun. 46, 220–223.
7. Beck, G., Habicht, G.S., Benach, J.L., and Coleman, J.L. (1985) J. Infect. Dis. 152, 108–117.
8. Yanagihara, Y., Kei-ichi, K., Yasuda, S., Kobayashi, S., Mifuchi, I., Azuma, I., Yamamura, Y., and Johnson, R.C. (1984) Microbiol. Immunol. 28, 535–544.
9. Joseph, R., Holt, S.E., and Canale-Parola., E. (1973) J. Bacteriol. 115, 426–435.
10. Heymer, B., Seidl, P.H., and Schleifer, K.H. (1985) In Immunology of the Bacterial Cell Envelope (D.E.S. Stewart-Tull, and M. Davies, Eds), pp. 11-25. John Wiley and Sons, Ltd., New York.
11. Kolenbrander, P.E., and Ensign, J.C. (1968) J. Bacteriol. 95, 201–210.
12. Stewart-Tull, D.E.S. (1980) Ann. Rev. Microbiol. 34, 311–340.
13. Spector, T. (1978) Anal. Biochem. 86, 142–146.
14. Umemoto, T., Ota, T., Sagawa, H., Kato, K., Takada, H., Tsujimoto, M., Kawasaki, A., Ogawa, T., Harada, K., and Kotani, S. (1981) Infect. Immun. 31, 767–774.
15. Markwell, M. (1982) Anal. Biochem. 125, 427–432.
16. Beck, G., Habicht, G.S., Benach, J.L., and Miller, F. (1986) J. Immunol. 136, 3025–3031.
17. Johnson, R.C., Hyde, F.W., and Rumpel., C.M. (1984) Yale J. Biol. and Med. 57, 529–537.
18. Stewart-Tull, D.E.S. (1985) In Immunology of the Bacterial Cell Envelope (D.E.S. Stewart-Tull, and M. Davies, Eds), pp. 47–65. John Wiley and Sons, Ltd., New York.
19. Oppenheim, J.J., Togawa, A., Chedid, L., and Mizel, S.B. (1980) Cell. Immunol. 50, 71–81.
20. Vacheron, F., Guenounou, M., and Nauciel, C. (1983) Infect. Immun. 42, 1049–1054.
21. Otha, M. (1981) Nippon Ika Daigaku Zasshi. 48, 402–409.
22. Beck, G., Habicht, G.S., Benach, J.L., Coleman, J.L., Lysik, R.M., and O'Brien, R.F. (1986) Zbl. Bakt. Hyg. A. 263, 133–136.