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Mitogenic Activity of Staphylococcal Peptidoglycan

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Staphylococcus aureus peptidoglycan displayed a marked dose-dependent mitogenic activity for mouse splenocytes and human peripheral blood lymphocytes in vitro, as measured by increased [^{3}H]thymidine incorporation. Similarly it was mitogenic for athymic nude mouse spleen cells, whereas no blastogenic effect was observed in T cell-enriched and B cell-depleted mouse lymphocyte cultures. These data demonstrate that peptidoglycan-responding cells in mouse spleen cell cultures are B lymphocytes.

Peptidoglycans (PG), heteropolymers present in cell walls of bacteria, actinomycetes and blue-green algae (cyanobacteria) (25) have been shown to possess numerous biological activities (14, 24), including immunopotentiating properties (15, 16, 18, 19), reticuloendothelial system stimulation (8), complement activation (1, 13, 22), and immunosuppressive effect (9).

Mitogenicity for mouse, rabbit, and human lymphocytes was reported for PG from *Nocardia* species, *Escherichia coli*, *Bacillus megaterium*, and PG-rich cell wall fraction from *Listeria monocytogenes* (4, 5, 6). All these PG have direct interpeptide bridges typical for gram-negative and gram-positive rods, consisting of D-alanyl-(D)-*meso*-diaminopimelic acid (D-Ala-*meso*-DAP). Peptide subunits of PG from gram-positive cocci lack *meso*-DAP and display great diversity in amino acid composition (25). Their mitogenic activity has not been reported. PG from *Micrococcus lysodeikticus* did not induce blastogenic transformation of mouse and rabbit lymphocytes (6), and in another study PG from *Staphylococcus aureus* failed to stimulate human peripheral blood lymphocytes (12).

The data reported in this paper demonstrate that staphylococcal PG is mitogenic for mouse splenocytes and human peripheral blood lymphocytes, and the responding cells in mouse splenocyte cultures are B lymphocytes.

MATERIALS AND METHODS

Mice. Female BALB/c mice 6 to 7 weeks old were obtained from Ace Animals, Inc., Boyertown, Pa., and fed a standard diet ad libitum. Female athymic nude mice (BALB/c background) 4 to 5 weeks old were obtained from Sprague Dawley, Madison, Wis. Female Swiss adrenalectomized mice 6 weeks old were obtained from Taconic Farms, Inc., New York.

Mitogens. PG was isolated from purified *S. aureus* 3528 and *S. aureus* 845 cell walls by the trichloroacetic acid extraction method as described elsewhere (10). It was suspended in sterile phosphate-buffered saline (PBS) solution and subjected to 60 min of ultrasonic treatment at kHz (8-watt output) in a W/185 Sonifier (Branson Ultrasonics Co., Plainview, N.Y.), and then heated at 70°C for 60 min and tested for sterility.

Phytohemagglutinin-P (PHA) (Difco Laboratories, Detroit, Mich.) was used as a control T cell mitogen for mouse splenocytes, and PHA-purified (Wellcome Research Laboratories, Beckenham, England) and concanavalin A (Calbiochem, San Diego, Calif.) for human lymphocytes. *E. coli* O127:B8 lipopolysaccharide (LPS), prepared by a phenol-water extraction method (Sigma Chemical Co., St. Louis, Mo.), was used as a control B cell mitogen.

Mitogenicity assays. Mouse spleen cell suspensions were prepared in RPMI 1640 medium (Microbiological Assoc., Walkersville, Md.) supplemented with 5% heat-inactivated fetal bovine serum (Flow Laboratories, Inc., Rockville, Md.) and an antibiotic-antimycotic mixture (Grand Island Biological Co. [GIBCO], Grand Island, N.Y.). Erythrocytes were lysed by hypotonic shock, and remaining splenocytes were washed with medium. Cultures (1 ml each) containing 10^6 viable cells were established in 24-well tissue culture plates (Linbro Scientific, Inc., Hamden, Conn.). An appropriate concentration of PG, PHA, or LPS was added (0.1 ml/well), and the cultures were incubated at 37°C in a humidified chamber containing 5% CO_2 and 95% air. After 24 h, each culture was pulsed with 1 μCi of [^{3}H]thymidine (New England Nuclear Corp., Worcester, Mass.) and incubated for additional 18 h. The cells were collected on glass microfiber filters (Whatman GF/A), dried, and suspended in 10 ml of Liquiscint (National Diagnostics, Parsippany, N.J.), and radioactivity was determined in a Beckman LS-330 liquid scintillation counter.

Human peripheral blood lymphocytes were obtained from normal healthy donors by Ficoll-Hypaque density-gradient centrifugation (2) and then were washed three times with Hanks balanced salt solution

(BSS; GIBCO). This method routinely yielded suspensions containing over 95% of mononuclear cells. The cells were suspended in Eagle minimal essential medium (GIBCO) supplemented with penicillin-streptomycin solution and 10% fetal bovine serum (Microbiological Assoc.). Cultures (0.2 ml each) containing 2.5 $\times 10^5$ cells were established in 96-well, flat-bottom Linbro plates, and appropriate concentrations of stimulants were added (10 μl/well). Cultures were incubated for 5 days as described above and pulsed with 1 μCi of [^{3}H]thymidine per well for the final 4 h. The cells were harvested on glass filters (Whatman 934AH) with an automated harvester (MASH II, Microbiological Assoc.), and radioactivity was determined as described above.

The results are expressed as stimulation indexes representing the ratio of average counts per minute for cultures treated with mitogen to average counts per minute for control cultures (without mitogen). Quadruplicate cultures were prepared, and each experiment was repeated several times.

B and T lymphocytes. Spleens from athymic nude mice were used as a source of B lymphocytes. T lymphocytes were obtained from spleen cells of normal BALB/c mice and fractionated on glass wool and nylon wool columns exactly as described by Julius et al. (17).

PG toxicity assay. Possible endotoxin contamination of PG preparations was assayed in adrenalectomized mice as described by Chedid et al. (3). Five days after adrenalectomy, mice (24) were injected with various doses of either PG or *E. coli* LPS, and mortality was recorded after 24 h. The 50% lethal dose (LD_{50}) was calculated by the method of Reed and Muench (23).

RESULTS

Toxicity of PG. Possible contamination of PG preparations with endotoxins, potent B cell mitogens, was assayed in adrenalectomized mice, which are extremely sensitive to LPS (3). The LD_{50} of PG was over 3,000 times higher than that of *E. coli* LPS (Table 1), thus ruling out endotoxin contamination of PG preparations employed in this study.

Mitogenicity of PG for murine splenocytes. Incubation of mouse spleen cells with graded concentrations of PG resulted in a marked dose-dependent stimulation of the cells as measured by the increase in [^{3}H]thymidine incorporation (Table 2). Stimulation index obtained with 400 μg of PG per culture, an optimal mitogenic concentration, was 25.7 for PG 3528 and 17.6 for PG 845, as compared to 15.9 for optimal PHA concentration and 56.6 for optimal LPS concentration.

TABLE 1. *Toxicity of PG and LPS assayed in adrenalectomized mice*[a]

Prepn	LD_{50} (μg)
PG	316
LPS	0.1

[a] Evaluated on 24 mice 24 h after PG 3528 or LPS challenge.

TABLE 2. *Mitogenic effect of PG on murine splenocytes in vitro*[a]

Stimulant added	Concn (μg/culture)	Stimulation index[b]
PG 3528	1	1.8 ± 0.1
	10	4.0 ± 0.3
	25	7.0 ± 0.3
	50	9.7 ± 0.4
	100	12.4 ± 0.4
	400	25.7 ± 0.9
	1,000	21.6 ± 0.4
PG 845	10	2.9 ± 0.2
	100	8.9 ± 0.3
	400	17.6 ± 0.2
	1,000	15.7 ± 0.4
PHA	1:50[c]	15.9 ± 1.1
LPS	100[d]	56.6 ± 2.0

[a] Cultures of 10^6 normal BALB/c mouse spleen cells were incubated with the indicated material for 24 h and pulsed with [^{3}H]thymidine for an additional 18 h.

[b] Stimulation index = mean counts per minute for treated cultures/mean counts per minute for control cultures ± standard error as determined from eight cultures, in two experiments. Background counts per minute (control cultures) = 1,662.

[c] Value indicates 1:50 dilution of stock preparation (optimal mitogenic concentration).

[d] Optimal mitogenic concentration.

Mitogenicity of PG for human lymphocytes. Incubation of human peripheral blood lymphocytes with PG resulted in a dose-dependent stimulation of the cells (Table 3) similar to that obtained for murine splenocytes. Optimal mitogenic concentration of PG from both strains studied was also 400 μg/ml, and the level of stimulation was similar to that induced by optimal doses of PHA or concanavalin A. LPS was not mitogenic for human lymphocytes, probably due to its toxicity as judged by a very low thymidine incorporation in LPS treated cultures compared to untreated controls. This confirms previous observations by other authors (20, 21).

Class of PG-responding cells. Spleen cells from athymic nude mice were used as a source of B lymphocytes. These cells were also stimulated by PG in a dose-dependent manner, and the stimulation indexes were higher than the corresponding values for normal spleen cell cultures (Table 4). Stimulation level of nude mouse splenocytes with LPS, a B cell mitogen, was of about the same magnitude as stimulation of normal spleen cells, whereas there was virtually no response to PHA, a T cell mitogen, thus confirming the lack of functional T lymphocytes in the nude mice used in these studies.

TABLE 3. *Mitogenic effect of PG on human peripheral blood lymphocytes*[a]

Stimulant added	Concn (μg/ml)	Stimulation index with the following lymphocyte donors:[b]		
		P.P.	D.A.	D.R.
PG 3528	1	NT[c]	4.4 ± 0.2	1.5 ± 0.1
	10	NT	11.9 ± 0.2	9.5 ± 0.3
	50	NT	15.2 ± 0.8	9.7 ± 0.5
	100	NT	18.8 ± 0.9	10.4 ± 0.5
	400	NT	20.4 ± 0.6	12.0 ± 0.6
	1,000	NT	19.4 ± 0.7	11.2 ± 0.6
PG 845	1	1.4 ± 0.1	1.5 ± 0.1	1.4 ± 0.1
	10	6.4 ± 0.5	7.9 ± 0.2	6.2 ± 0.3
	50	17.9 ± 0.4	13.9 ± 0.8	12.0 ± 0.8
	100	28.2 ± 1.2	14.3 ± 0.7	14.9 ± 0.8
	400	NT	18.1 ± 1.1	19.6 ± 0.6
	1,000	NT	16.5 ± 0.5	15.7 ± 0.9
PHA	0.12[d]	38.7 ± 2.9	14.8 ± 0.7	21.9 ± 0.5
ConA	0.3[d]	16.2 ± 1.7	17.4 ± 0.8	19.4 ± 0.7
	0.6	25.8 ± 2.1	17.0 ± 0.4	20.8 ± 0.7
	1.5	26.3 ± 2.0	9.4 ± 0.7	20.0 ± 1.8
LPS	1	NT	0.8 ± 0.06	1.0 ± 0.07
	10	NT	0.8 ± 0.08	1.0 ± 0.09
	50	NT	0.7 ± 0.04	0.9 ± 0.1
	100	NT	0.6 ± 0.04	0.8 ± 0.04
	400	NT	0.2 ± 0.03	0.2 ± 0.03

[a] Cultures of 2.5×10^5 lymphocytes from normal donors were cultured in 0.2 ml of medium with the indicated material for 5 days and pulsed with [^{3}H]thymidine for 4 h. ConA, Concanavalin A.
[b] Determined from four cultures; background counts per minute for donor P.P. = 1,099; D.A. = 2,252; D.R. = 1,478; stimulation indexes were calculated as in Table 2.
[c] NT, Not tested.
[d] Optimal mitogenic concentration(s).

TABLE 4. *Mitogenic effect of PG on spleen cells of nude mice*[a]

Stimulant added	Concn (μg/culture)	Stimulation index[b]
PG 3528	1	4.0 ± 0.1
	10	6.5 ± 0.8
	50	13.0 ± 0.5
	100	17.1 ± 0.7
	400	27.4 ± 0.4
	1,000	29.1 ± 0.4
PHA	1:50[c]	0.8 ± 0.03
LPS	100[d]	49.4 ± 0.6

[a] Cultures of 10^6 spleen cells from athymic nude mice were incubated as described in footnote *a* to Table 2.
[b, c, d] Values determined from four cultures; background counts per minute = 2,274; other details as in footnotes *b*, *c*, and *d*, respectively, to Table 2.

TABLE 5. *Lack of mitogenic effect of PG on murine T lymphocytes*[a]

Stimulant added	Concn (μg/culture)	Stimulation index[b]
PG 3528	1	1.0 ± 0.1
	10	1.2 ± 0.1
	50	1.5 ± 0.2
	100	1.5 ± 0.2
	400	1.4 ± 0.1
	1,000	1.1 ± 0.1
PHA	1:50[c]	94.1 ± 2.1
LPS	100[d]	3.7 ± 0.6

[a] Cultures of 10^6 splenocytes enriched in T lymphocytes by a nylon wool column adherence method were incubated as described in footnote *a* to Table 2.
[b, c, d] Four cultures, background cpm = 358; other details as in footnotes *b*, *c*, and *d*, respectively, to Table 2.

The blastogenic responsiveness of mouse T lymphocytes to PG was then studied. PG was not mitogenic for T cell-enriched preparations of normal spleen cells (Table 5). The mitogenic response of these cells to PHA was almost six times higher than that of unfractionated splenocytes, whereas their blastogenic responsiveness to LPS was very low (6.5% of the response of unfractionated cells), thus indicating an overwhelming predominance of T lymphocytes in column-purified cells employed in these studies.

DISCUSSION

The results demonstrate that staphylococcal PG is a potent mitogen for human and mouse lymphocytes. Similar stimulations were obtained with PG from both *S. aureus* strains studied, and the responding cells in mouse spleen are B lymphocytes. The effect can be attributed to PG alone and not to any endotoxin contamination of PG preparations, as revealed by toxicity tests in adrenalectomized mice, its mitogenicity for human lymphocytes (while LPS is not active), and as previously shown by chromatography (10) and pyrogenicity tests in which PG solubilized by ultrasonic treatment was not pyrogenic for rabbits in doses of 1 mg/kg and lysozyme or lysostaphin digestion of particulate PG decreased its pyrogenicity (11).

Mitogenic activity of staphylococcal PG is similar to that previously reported for PG from *Nocardia* and gram-negative and gram-positive rods (4, 6). There are still conflicting reports in the literature concerning mitogenically active principle of PG and its minimal chemical structure required for mitogenic activity. In this study we employed a large-molecular-weight particulate PG preparation, and staphylococcal PG solubilized by ultrasonic treatment was not mitogenic for mouse and human lymphocytes (Dziarski, unpublished data). This may explain the failure of Forsgren and co-workers (12) to induce blastogenic transformation of human lymphocytes by treatment with staphylococcal PG. They did not indicate, however, the extent of solubilization of their PG preparations. Also, PG from *E. coli*, *N. rubra* and *N. opaca* solubilized by lysozyme digestion were not mitogenic (4, 6), and adjuvant active muramyl dipeptide, a synthetic analog of basic PG structure, possessed weak, if any, blastogenic activity for mouse lymphocytes (26, 27), although it was distinctly mitogenic for guinea pig lymphocytes (27). However, fragments of *Nocardia* PG solubilized by lysostaphin treatment possessed blastogenic activity (4), indicating that intact glycan strands substituted by tetra- or tripeptides are required for mitogenic activity of PG. It is still not known whether this is the case with PG from *S. aureus* and other gram-positive cocci possessing a different structure of a peptide portion of PG. However, recent studies with synthetic muramyl dipeptide revealed that it can produce a distinct mitogenic effect in mouse splenocytes cultured for 4 or 5 days in serum-free medium supplemented with 2-mercaptoethanol (7). These data show that mitogenic activity of PG is a complex issue, depending not only on the structure of PG preparation but also on animal species and culture conditions.

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